STUDY OF SECRETORY PROTEIN (S) OF *MYCOBACTERIUM TUBERCULOSIS*: MOLECULAR CHARACTERIZATION AND ANALYSIS OF IMMUNOLOGICAL PROPERTIES WITH SPECIAL REFERENCE TO APPLICATION IN SERODIAGNOSIS AND UTILITY OF VACCINATION AGAINST TUBERCULOSIS

THESIS

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DILEEP TIWARI

CERTIFICATE

(As required under ordinance of the Bundelkhand University)

This is to certify that the work embodied in this thesis entitled ***"Study of secretory protein (s) of Mycobacterium tuberculosis: Molecular characterization and analysis of immunological properties with special reference to application in serodiagnosis and utility of vaccination against tuberculosis"*** has been carried out by **Mr. Dileep Tiwari** under our guidance and supervision for the degree of Doctor of Philosophy in Biotechnology at Department of Biotechnology, J.C. Bose Institute of Life science, Bundelkhand University, Jhansi (U.P.) India.

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(Dileep Tiwari)

Dedicated To Humanity

CONTENTS

ABBREVIATIONS

CFP	Culture Filtrate Protein
Ag	Antigen
Ab	Antibody
AP	Alkaline Phosphates
PBS	Phosphate buffered saline
PB	Phosphate buffer
PBT	Phosphate Buffered with Tween-20
PBST	Phosphate Buffered Saline with Tween-20
EDTA	Ethylene Di-amine Tetra-acetic Acid
AIDS	Acquired Immuno Deficiency Syndrome
AFB	Acid Fast Bacteria
BSA	Bovine Serum Albumin
BCG	Bacillus Calmette Gurein
CFA	Complete Friends Adjuvant
CFU	Colony Forming Unit
DHT	Delayed Type Hypersensitive T cell
DNA	Deoxyribonucleic Acid
DOTS	Direct Observed Treatment Short course
E. coli	*Escherichia coli*
ECG	Electrocardiogram
ELISA	Enzyme Linked Immunosorbent Assay
Gm	Gram
HIV	Human Immunodeficiency Virus
Hrs	Hours
HRP	Horse Radish Peroxidase
IFA	Incomplete Friends Adjuvant
IFN-γ	Interferon-gamma
RNA	Ribonucleic Acid
RT	Room Temperature

IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
KDa	Kilo Dalton
Kb	Kilo base pair
Lt	Litre
LB	Luria Bertani
Lip	Liposome
LAM	Lipoarabinomannan
Min	Minutes
mg	Milligram
µg	Microgram
M.TB	*Mycobacterium tuberculosis*
mM	Milli moles
MDR	Multi-Drug Resistance
ml	Millilitre
µl	Microlitre
NC	Nitrocellulose
NHS	Normal Human Sera
ng	Nanogram
OD	Optical Density
OPD	Out Patients Department
PPD	Purified Protein Derivatives
PAGE	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction
RBC	Red Blood Corpuscles
rpm	Revolutions per minute
RE	Restriction Endonuclease
RNTCP	Revised National Tuberculosis Control Program

SDS	Sodium Dodecyl Sulphate
STD	Sexually Transmitted Disease
TAE	Tris Acetate EDTA
TE	Tris EDTA
TMB	Tetra Methyl Benzedine
WHO	Word Health Organization
MISP	Most Immunoreactive Secretory Protein
SP	Secretory Protein
CSP	Cocktail of Secretory Protein

1

Introduction

Tuberculosis is one of the oldest and most wide spread life-threatening infectious diseases of mankind. It is characterized by fever, weight loss, raised level of acute phase reactants and necrosis in lesions.

It has been estimated that every third person on this earth is infected with *M. tuberculosis*, infecting nearly 1.6 billion people of which 20 million have active disease, with eight million new cases and approximately 2-3 million deaths annually world wide *(*WHO report, 2004*)*. India alone has nearly one third of the total global burden of tuberculosis, and the disease is one of the India's most important public health problems. Every day in India more than 20,000 people become infected with the tubercle bacillus, more than 5,000 develop the disease, and more than 1,000 die from Tuberculosis.

In India, tuberculosis kills 14 times more people than all the other tropical diseases combined, 21 times more than malaria, and 400 times more than leprosy. Every year, another 2 million people develop tuberculosis; nearly one million of them are highly infectious, sputum positive. Two such cases develop every minute. Further, in recent years there has been an upsurge in the incidence of the disease mainly due to the emergence of multidrug resistant strains of *M. tuberculosis* (MDRTB) and its association with the HIV infection leading to spread of disease in immuno-compromised and AIDS positive individuals. Globally, there are about 4.6 million cases dually infected with HIV and tuberculosis *(*WHO report, 2002; 2004*)*. Tuberculosis thus, has become a major concern worldwide. If specific control measures are not taken with increased commitment and action at national and International levels, tuberculosis will claim about 35 million more lives in the next decade and

there will be about 150 million new cases of active tuberculosis. Such a vast epidemic creates challenges as it raises the demand for public health solutions. A delayed or missed diagnosis of tuberculosis is also one of the leading causes, which contribute to *M. tuberculosis* transmission.

Currently, more than 15 anti-*Mycobacterium* drugs are available for tuberculosis patients and are indispensable to prevent the progression of the disease especially those caused by MDR strains of *M. tuberculosis*. Thus, there is an urgent need to develop strategies to effectively tackle the threat of *Mycobacterium* to the mankind.

Since, the identification of *Mycobacterium* by Koch in 1882 as the causative agent of the disease, efforts have been made to develop suitable prophylactic and chemotherapeutic agent(s) for the treatment of disease. In the past, several killed and live vaccines were developed to protect against the disease. However, the only vaccine that is still in use is derived from the bovine strain of *Mycobacterium* i. e. *M. bovis*. This strain was attenuated for over thirteen years and was termed as Bacilli Calmette Gurein (BCG) vaccine (Calmette, 1927). Since then BCG vaccine has been successfully used for prophylaxis against tuberculosis. It remains to be the most wildly and most controversial of all vaccines used today. The protective efficacy of BCG vaccine has been highly variable ranges from 0-80% (Fine, 1989; Baily, 1990). Moreover, it has been observed that BCG vaccine is least effective in controlling the disease in the regions/areas where the frequency of the disease is maximum (Stanford, 1991). In addition, the BCG has been shown

to produce disseminated infection in the immunocompromised host such as AIDS patients, sometime even after thirty years of vaccination (Orme *et al.* 1993). Therefore, there is an urgent need to identify the protective component of *Mycobacteria* for the development of safe, stable and more effective vaccine against tuberculosis.

It is evident from several studies that resistance against tuberculosis is mediated by cellular arm of the immune system *(Leveton et al.,* 1989; Orme *et al.,* 1993; Turner *et al.,* 2000; Kanaujia *et al.,* 2004). Hence, the antigen for vaccine development should be selected on its ability to induce the cell mediated immune response. In the past, attempts have been made to investigate the immunogenic potential of different components of *Mycobacterium.* Several components *viz. Mycobacterial* cell wall, rRNA and mannosides have been evaluated for their immunoprotective potential (Mehta, 1996; Bouquet and Negre, 1923; Youmans and Youmans, 1966a; Pancholi *et al.,* 1989; Singh and Khuller, 1993; Hetzel *et al.* 1998; Oliver *et al.,* 2000; Gennaro, 2000; Leander *et al.,* 2005; Kwasi *et al.,* 2005).

Secretory proteins of *M. tuberculosis* appear as culture filtrate proteins (CFPs) in the culture medium in which *M. tuberculosis* is grown. Mechanism of secretion of CFPs is not clearly known. There are approximately 200 proteins found in culture filtrate of *M. tuberculosis* (Anderson, 1994; Berthet *et al.,* 1998; Kamath *et al.,* 1999; Sonnenberg and Belisle, 1997; Karin *et al.* 1998; Kanaujia *et al.,* 2004; Spencer *et al.,* 2004; Young *et al.,* 2004; Sable *et al.,*

2005). Some of these proteins are associated with cells, therefore, the definition of CFP is an operational one.

Many research groups actively studied CFPs. Since many CFPs are recognized by the sera of TB patients, it has also been postulated that live attenuated *M. tuberculosis* vaccines are better than those made from heat-killed cells because during growth in the host, M. tuberculosis releases CFPs that stimulate host immune mechanisms (Andersen, 1994).

Many of the proteins found in the culture filtrate viz. SodA, KatG, and GlnA (glutamine synthase), do not have leader sequences that are usually involved in protein secretion, but the fact that they were released from cells early in growth suggested that this localization was physiological and not dependent on cell lysis (Daffe and Draper, 1998. Braunstein and Belisle, 2000; Brennan and Nikaido, 1995; Sonnenberg and Belisle, 1997; Tullius *et al.,* 2001;Smith Issar, 2003; Pym *et al.,* 2001). However, experiments suggest that proteins highly expressed in *M. tuberculosis* are GlnA and SodA (Piddington *et al.,* 2001; Dussurget, 2001;Harth and Horwitz, 2003). They are very stable and found in culture filtrates in early period of incubation, while less abundant intracellular proteins or those that are unstable are not found extracellularly (Molle *et al.,* 2000). These results strongly suggest that the presence of many proteins in culture filtrates, especially those with missing leader sequences, is caused by bacterial leakage or lysis.

The M. Tb secreted active proteins, which are missing in non-tuberculous *Mycobacteria* have proved as a promising not only for diagnostic marker, vaccine candidate but also in understanding the *Mycobacterial* evasion of protective immunity in susceptible individuals. Many such proteins identified and characterized from the M. TB complex include 38 KDa, 30/31KDa, 40 KDa, 42 KDa, SOD, 30 KDaMSP, 85B, ESAT-6, and CFP10 (Sonnenberg and Belisle 1997; Manca *et al.,* 1997; Karin *et al.,* 1998; Gennaro 2000; Silva *et al.,* 2003; Young *et al.,* 2004; Lanbo *et al.*, 2004; Ayman *et al.,* 2005). However, little information is known regarding the possible role of low molecular mass M. TB secretory proteins in immunity against tuberculosis, with emphasis on their immuno-modulatory action and the potential involvement in *Mycobacterial* subversion of the host immune defense.

Another important area of significant concern is the diagnosis of the disease. Although several studies have been conducted and many are still in progress for diagnosis of disease during the early stage of infection, which can distinguish *M. tuberculosis* infection from other atypical *Mycobacteria* is a challenging task worldwide.

The current methods used in clinical laboratories depend on microscopy and culture that usually takes 6-8 weeks to report negative / positive results. The isolation of organism from CSF has been disappointingly infrequent (Dingley 1979), time consuming, and lacks sensitivity (Annamma *et al.*, 1990; Clarridge *et al.,* 1993;Kadival *et al.,* 1987). These methods have several limitations both in terms of its sensitivity and specificity (Garg *et al.,* 2003). Thus the bottom line in the effective treatment still remains i. e. the early diagnosis of the

disease that could help in taking remedial measures far more early than is done at the moment. Another most commonly used procedure for diagnosis is 'Montoux test' which doesn't make a conclusive evidence of active disease, while negative tests do not exclude it.

Several attempts have been made in the past to develop newer tools for the timely, accurate and specific diagnosis in the early stages of disease by developing the tests based on immunological and molecular methods to reduce the time of diagnosis, increased sensitivity and specificity. Although rapid culture techniques such as Radiometric liquid (BACTEC), biphasic (MB chek) culture system and Alamar blue assays have improved both the recovery rates and speed of isolation, but these systems still can not influence beside decision making (Garg *et al.,* 2003; www.nejm.org on March 27, 2006; Dingley, 1979; Annamma *et al.,* 1990, Clarridge *et al.,* 1993, Kadival *et al.,* 1987).

The introduction of fibreoptic bronchoscope has rendered lung biopsy as well as bronchial lavage and brushing a simple and safe procedure but unfortunately the equipments are not universally available (Zavala, 1975; Miro *et al.,* 1997; Alan *et al.,* 2002;Griffith *et al.,* 2004).

Recently, several tests have been developed employing the r-DNA technology to develop the kits based on polymerase chain reaction (PCR) in diagnosis of tuberculosis. A number of investigators have reported the detection of specific sequence for *M. tuberculosis* directly in clinical specimen by PCR (Miller *et al.,* 1994; Bisen *et al.,* 2003). This technique is quite sensitive and by making

proper selection of gene sequences, it is possible to accurately establish the diagnosis *M. tuberculosis* complex in the clinical samples. But despite its sensitivity and specificity, this method is highly expensive e.g. one Amplicon PCR test costs~ $15 (Bennedsen *et al.,* 1996). Hence, the application of PCR based diagnosis is the best but also very costly. Moreover, the amplification of dead bacterial DNA and absence of amplifiable *M. tuberculosis* DNA from blood pose a serious problem in the diagnosis (Mullis and Faloona, 1987; Mileler *et al.,* 1994; Garg *et al.,* 2003; Bisen *et al.,* 2003).

Several serodignostic tests have been developed for the diagnosis of disease (Grange and Laszlo, 1990;Gennaro, 2000; Nair *et al.,* 1992;Garg *et al.,* 2003; Esther *et al.,* 2004;Young *et al.,* 2004;Tiwari *et al.,* 2005). Many workers have attempted to isolate the species specific antigen for use in diagnostic tests (Manca *et al.,* 1997b;Kadiwal *et al.,* 1994; John *et al.,* 1998;Griffin *et al.,* 1991; Daleine, 1995;Sudha *et al.,* 2000; Esther *et al.,* 2004;Tiwari *et al.,* 2005), but this task has proved very difficult, because of two reasons: firstly, specific antigenic determinants often occur on the same protein molecule as shared antigen, therefore making it very difficult to purify them by sensitive techniques viz. affinity chromatography etc. Secondly, an antigenic determinant, many a times may be shared among different species of genera *Mycobacterium* that make the correct diagnosis of *M. tuberculosis* difficult. Also the given determinant may be present on a range of molecules of differing physiochemical properties. Thus preparative techniques based on such difference (gel-filtration & ion exchange chromatography) have not proved very useful (Grange 1988 a and b).

The rapid diagnostic test for the detection of antigen(s) in patients with tuberculosis may be the best choice for diagnosis of tuberculosis, as false positive results have been observed in antibody based diagnostic tests due to exposure of environmental Non-tuberculous *Mycobacteria* (NTM) or prior BCG vaccination (Fine 1995).

Thus the ultimate goal of biomedical research in TB around the world should be to lessen the public health burden of this disease by developing improved, specific and cost effective prophylactic diagnostic tools (Eunice *et al.* 2003) to combat the menace of the disease.

The present study was undertaken with the aim to identify the major immunoprotective and immunodiagnostic antigens of *M. tuberculosis* for the development of effective vaccine and / or diagnostic tool. The secretory protein (s) antigens of *M. tuberculosis* were identified in order to develop a rapid, cost effective, sensitive and specific test for the detection of *M. tuberculosis* antigens and / or antibodies (IgG, IgM, IgA) in patients suffering from active tuberculosis infection. The choice of specimens included the CSF for Tuberculosis Meningitis, serum for pulmonary and other extra pulmonary tuberculosis and processed tissue biopsy for extra-pulmonary tuberculosis.

2

Review of Literature

2.0 General Introduction

Tuberculosis (TB) is a chronic infectious disease caused by *Mycobacterium tuberculosis.* It is one of the oldest human afflictions which has plagued mankind throughout recorded and archeological history leading to infectious killer of youth, children and adults and second most common infectious disease worldwide (WHO report 1995), despite the global use of live attenuated vaccine and several antibiotics. It has been estimated that there are nearly 1.7 billion individuals infected with *Mycobacterium tuberculosis* throughout the world, approximately 20 million of these are active cases and three million die each and every year leading to eight million new cases annually and three million of these are infectious (WHO report 2002). In India, out of the 950 million inhabitants, 300 million are infected with *M. tuberculosis*, 12 million have active tuberculosis, three million are infectious and half a million die each year (WHO 2002, and 2004).

One third of world's population is currently infected (Kochi *et al.,* 1994; WHO report, 2003) with *M. tuberculosis* in which 20 million of these are infected with active cases. However these can further infect 50-100 million people (Largely children) annually.

The mortality due to disease is approximately 3 million annually; at least 80% of those are in the developing countries (Smith Issar, 2003). Thus, tuberculosis is still a major cause of disease and mortality whose elimination will be impossible as long as poverty, overpopulation and malnutrition is concerned (Smith, 2003). TB is now becoming the leading cause of death

among HIV positive people where it kills much more rapidly with a fatality of 80% population.

The epidemic of HIV infection has radically changed the epidemiology of tuberculosis. Because of its ability to destroy the immune system, HIV has emerged as the most significant risk factor for progression of dormant TB infection to clinical disease (Selwyn *et al.,* 1989).

2.1 ORIGIN:

The origin of *Mycobacterium tuberculosis*, the causative agent of TB, has been subject of much recent investigations and it is thought that the bacteria in the genus *Mycobacterium,* like other actinomycetes, were initially found in soil and that some species evolved to live in mammals. The domestication of cattle, thought to have occurred between 10,000 and 25,000 years ago, would have allowed the passage of a Mycobacterial pathogen from domesticated livestock to humans and in this adaptation to a new host. The bacterium would have evolved to the closely related *M. tuberculosis.* Specifically, it has been hypothesized that *M. bovis*, which causes a TB-like disease in cattle was the hypothetically evolutionary precursor of *M. tuberculosis* (Stead, 1997). This hypothesis is now considered doubtful in the light of new data, since it was formulated before the genome in the *M. tuberculosis* complex including the human and animal pathogens *M. africanum, M. microti and M. Canetti* as well as *M. tuberculosis* and *M. bovis*, were characterized by DNA sequencing and related methods. These studies have shown greater than 99.9% similarity of DNA sequencing among the members of the *M.*

tuberculosis complex (Brosch *et al.,* 2002). But the existence of rare synonymous single nucleotide polymorphisms (sSNP) allows discrimination between these closely related bacteria. sSNP analyses suggest that *M. bovis* evolved at the same time as *M. tuberculosis* (Sreevatsan *et al.,* 1997) and a study of the distribution of deletion and insertions in the genomes of the *M. tuberculosis* complex provides strong evidence for the independent evolution of both *M. tuberculosis* and *M. bovis* from another precursor species, possibly related to *M. canetti* (Brosch *et al.,* 2002).

It is thought that the progenitor of the *M. tuberculosis* complex, comprising *M. tuberculosis*, *M. bovis* BCG, *M. africanum* and *M. microti* arose from a soil bacterium and that the human bacillus may have been derived from the bovine from following the domestication of cattle. The complex lacks inter strain genetic diversity and nucleotide changes are very rare.

This is important in terms of immunity and vaccine development as most of the proteins will be identical in all strains and therefore antigenic drift will be restricted. On the basis of the systematic sequence analysis of 26 loci in a large number of independent isolates, it was concluded that the genome of *M. tuberculosis* is either unusually inert or that the organism is relatively young in evolutionary terms.

2.2 Establishment of *Mycobacterial* infections

Since 1882, Robert Koch identified *Mycobacterium tuberculosis* as the causative agent of TB, *M. tuberculosis* can be best described as an obligate

aerobe, generally characterized by a long replication time and a cell wall containing abundant lipids and waxes that provide hydrophobic characters, acid fast properties and intracellular survival (Gebbardt *et al.,* 1996). There are five closely related *Mycobateria* grouped in the *M. tuberculosis* complex: *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti*, and *M. canetti* (Van Soolingen *et al.,* 1997; Van Soolingen *et al.,* 1998). Members of the *M. tuberculosis* complex can all cause the disease in humans, although *M. tuberculosis* is the most prevalent. The natural reservoir of *M. tuberculosis* and *M. africanum* is limited to humans and that of *M. microti* is mainly limited to small rodents (Kremer *et al.,* 1998). In contrast, *M. bovis* can cause disease in a wide range of wild and domestic animals as well as in humans (Brosch *et al.,* 2002; Morris *et al.,* 1994).

Transmission of TB in man usually occurs via air borne microscopic droplet nuclei (1-5 μm diameter) containing *M. tuberculosis*. The infectious droplet nuclei are inhaled and lodge in the pulmonary alveoli (Loudon and Roberts, 1967; Riley *et al.,* 1995), where, then the bacilli are phagocytosed by alveolar macrophages and remain in the phagosome of these cells (Armstrong and Hart, 1975). Following phagocytosis, *M. tuberculosis* replicates slowly but continuously, and is spread to the neighboring lung tissue and through lymphatic vessels to draining hilar lymph nodes (Frieden *et al.,* 2003).

It is not fully understood how *M. tuberculosis* can survive and replicate intracellularly in macrophages, which are cells that have the microbicidal armory to destroy most pathogens. However, *M. tuberculosis* seems to have

evolved mechanisms to survive most of the macrophage-effector functions. Some of these mechanisms involve the inhibition of the phagosome-lysosome fusion, where the bacilli have been found to retain a macrophage protein, called tryptophane aspartate-containing coat protein (TACO), on the surface of the phagosome, preventing their delivery to the lysosome (Fratazzi *et al.,* 1999), and to use complement receptors 1 and 3 for cell entry, which do not trigger oxidative burst (Schlesinger *et al.,* 1990; Wright and Silverstein, 1983). Other mechanisms of survival include degradation of reactive oxygen intermediates by catalase and superoxide dismutase produced by the bacilli, inhibition of apoptosis in infected macrophages (Fratazzi *et al.,* 1999), and down-regulation of some modulators of the host immunity such as interleukin 12 (IL-12) (Hickman *et al.,* 2002; Nau *et al.,* 2002), major histocompatibility complex (MHC) class II (Noss *et al.*, 2000), and interferon γ (IFN-γ), known to mediate activation of macrophages (Ting *et al.,* 1999).

After *M. tuberculosis* has entered the lungs, one of the four potential fates might occur (Schluger and Rom, 1998):

1) The initial host response can be completely effective in the killing and elimination of the bacilli, such that these individuals have no chance to develop TB.

2) The bacilli can grow and multiply immediately after infection, causing clinical disease (primary TB).

3) The bacilli may become dormant and never cause disease at all, resulting in a latent infection that is manifested only as positive tuberculin skin test results;

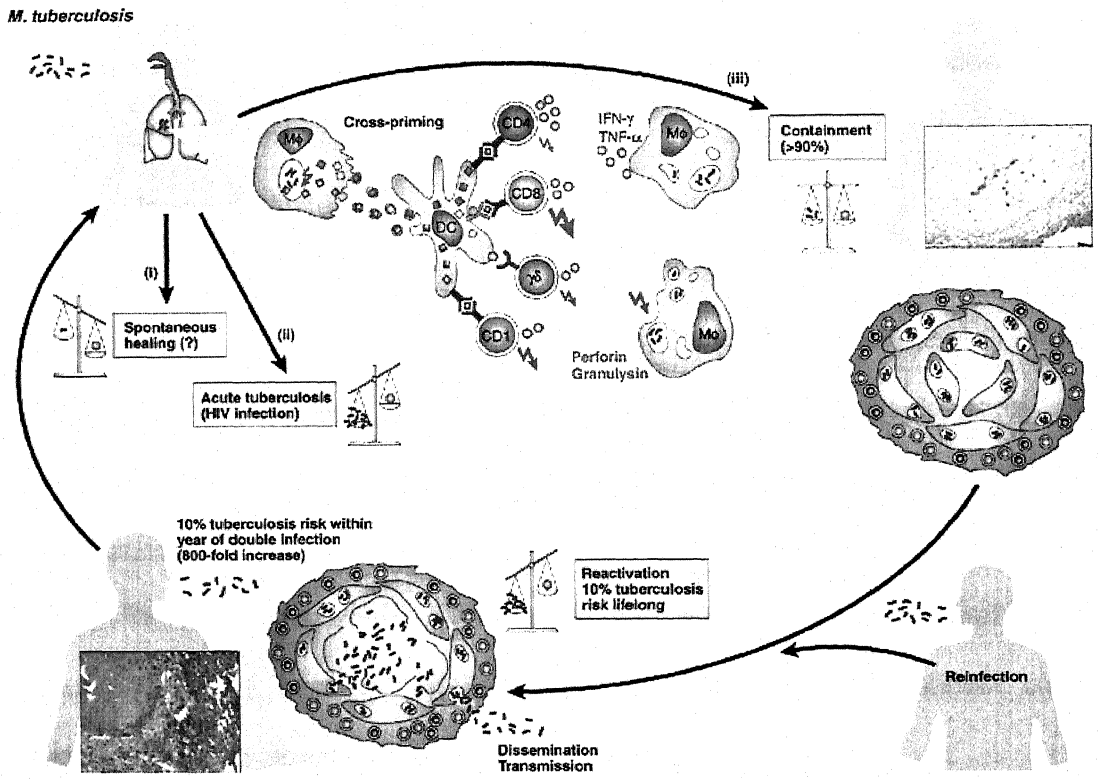


Figure 1: Different outcome of *M. tuberculosis* infection and underlying immune mechanisms. *M. tuberculosis* enters the host within inhaled droplets.

4) The dormant bacilli can eventually begin to grow, due to factors like Immunosuppression, with resultant clinical disease (reactivation TB).

2.3 History:

First credible understanding that tuberculosis might be due to infectious microorganisms was made in 1722 by Benjamin Martin of London, who proposed that the cause of tuberculosis was "animalcule or their seed inimicable to our Nature" that can be transmitted by "a Breath (a consumptive) emitted from his lungs that may be caught by a sound person" (Doetesch 1978; Castiglioni 1933). The tuberculosis appears to be as old as humanity itself. TB can be present in various forms, including one that attacks bone and causes skeletal deformities. Hard tissues like bone can be preserved for thousands of years, allowing almost certain identification of individuals with bone TB, who died more than 4000 years ago. Skeletal remains of prehistoric humans dating back to 8000BC, found in Germany have shown clear evidence of the disease.

More than a century after Laenmec's birth, Villemin performed experiments on rabbits, injecting infectious sputum and caseous material into healthy animals to produce disease. The studies conducted in 1868 and cited by major (1945), provided first convincing evidence of the infectious nature of tuberculosis. Gradually, the infectious nature of tuberculosis became more widely recognized. As early as 1699, Italy and later Spain enacted restrictive quarantine laws, while in Northern Europe; tuberculosis was not widely viewed as a public health problem.

The frequency of unearthed skeletons with apparent tuberculosis deformities in ancient Egypt suggests that the disease was common among that population. The discovery of similarly deformed bones in various Neolithic sites in Italy, Denmark and countries in the Middle East also indicates that TB was found throughout the world up to 4,000 years ago. (Smith, 2003). Ancient Hindu and Chinese writing have documented the presence of this disease.

The under developed world including India is still suffering with high TB morbidity and mortality rates as shown by the following statistics. The incidence of TB ranges from less than 10 per 100,000 in North America to 100 to 300 per 100,000 in Asia and Western Russia to over 300 per 100,000 in Southern and Central Africa. There is one death from TB every 15 sec (Over two million per year) and eight million people develop TB every year. Without treatment, up to 60 percent of people with the disease will die (Kaye and Frieden, 1996).

Essentially all these cases are in the Third World (World Health Organization. 2002), reflecting the poverty and the lack of healthy living conditions and adequate medical care (Waaler, 2002). This global crisis is compounded by the emergence of multidrug resistance in countries like the former Soviet Union, South Africa, and India, where some antibiotics are available but are of inferior quality or are not used for a sufficient time to control the disease according to recommended regimens (Iseman, 1994: O'Brien, 2001).

Unlike many infectious diseases the epidemic wave of TB measures and Centuries-long epidemiological information though incomplete, reflects the incidence and prevalence of disease over a period of two to three centuries. The wave from of the tuberculosis epidemic occurs by natural selection of susceptible persons and runs its course in about 300 years. (William and Dutt. 2002).

In England, the presence of epidemic wave began in the 16th century and probably reached its peak about 1780 as a result of the Industrial revolution and the growth of cities, which allowed the spread of disease from person to person. The epidemic then rapidly spread from England to other large-cities in Western Europe, reaching a peak in the early 1800s. In Eastern Europe the peaks came about 1870 and 1888 and by 1900 North American and South American epidemic waves had peaked. In the developing countries of Asia and Africa, the wave has not peaked yet. Thus, as a global phenomenon, the epidemic is declining in one geographic area while still rising or just reaching its peak in another.

Industrialization and overcrowding of cities can produce an epidemic of tuberculosis by bringing together large numbers of susceptible people and promoting transmission of *Mycobacterium tuberculosis* to new hosts.

TB morbidity and mortality rates due to TB steadily dropped during the 20th century in the developed world, aided by better public health practices and widespread use of the *M. bovis* BCG vaccine as well as the development of

antibiotics in the 1950s. This downward trend ended and the number of new cases started increasing in the mid-1980s. The major causes of this were increased homelessness and poverty in the developed world and the emergence of AIDS, with its destruction of the cell-mediated immune response in co-infected persons. Only by massive expenditures of funds and human resources mainly by directly monitored antibiotic delivery "miniepidemic" of new TB cases has been reversed in Europe and the United States (Frieden *et al.,* 1995).

Thus, TB is caused by bacterial, but environmental factors play a major role, an idea that Rene Dubos clearly rearticulated 50 years ago (Dubos and Dubos, 1952). To Dubos, purely medical solutions alone would not work to cure and prevent TB. Unfortunately, the events of the last half of the 20th century have shown how prescient he was. The antibiotic era, begun by the discovery of streptomycin by Schatz and Waksman in the 1940s and its use to treat TB and followed by the introduction of many other antibiotics like isoniazid, rifampin, and pyrazinamide that are useful against TB, has not eliminated the disease (Ryan, 1992). Likewise, the widespread use of BCG, an attenuated vaccine strain produced by the sequential passage of a virulent *M. bovis* strain by Calmette and Guerin in Paris in the 1920s, has not lowered the incidence of TB in recent years (Andersen, 2002) and there is more TB today than ever before (Waaler, 2002).

2.3.1 NON TUBERCULOUS MYCOBACTERIA

More than 25 *Mycobacterial* species other than *M. tuberculosis* can be found in specimens from humans. Some of these species may have quite a high

likehood of being human pathogens. They include rapidly growing *M. fortuitum* and *M. chelonae* and slowly *growing M. kansasii, M. avium, M. intracellular, M. scrofulaceum, M. xenopi, M. malmoense, M. simiae, M. szulgdi, M. marinum, M. haemophilum* and *M. ulcerans.*

2.4 FEATURES OF *MYCOBACTERIUM TUBERCULOSIS*

A characteristic feature of the *M. tuberculosis* includes its slow growth, dormancy, complex cell envelope, intracellular pathogenesis and genetic homogeneity. The generation time of *M. tuberculosis*, in synthetic medium or infected animals, is typically ~24 hours. This contributes to the chronic nature of the disease, imposes lengthy treatment regimens and represents a formidable obstacle for researchers. The state of dormancy in which the bacillus remains quiescent within infected tissue may reflect metabolic shutdown resulting from the action of a cell-mediated immune response that can contain but not eradicate the infection. As immunity wanes, through ageing or immune suppression, the dormant bacteria reactivate, causing an outbreak of disease often many decades after the initial infection. The molecular basis of dormancy and reactivation remains obscure but is expected to be genetically programmed and to involve intracellular signaling pathways.

2.4.1Growth

M. tuberculosis grows well on liquid and solid media such as: -

A. Liquid media

(i) Middlebrook 7H9 and 7H12B broth supplemented with 10% ADC (albumin dextrose and catalase) at 37° C.

(ii) Sautons media for 6-7 weeks at 37° C (Figure-2 a)

B. Solid media

(i) Middlebrook 7H10-7H11 agar supplemented with 10% OADC (Oleic acid, albumin dextrose and catalase) at 37° C

(ii) Lownstein Jenson (LJ) slants. (Figure –2 b)

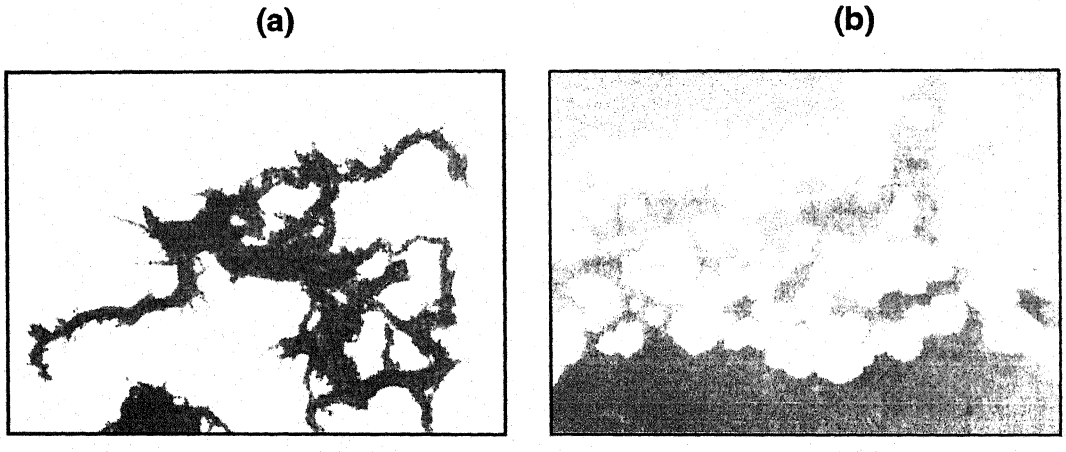


Figure 2: Growth of M. tuberculosis culture on (a) liquid medium and (b) on solid medium.

2.4.2 Pigmentation

Depending on the pigment produced as well as rate of growth (Runyon *et al.*, 1959) sub classifies the non-tuberculosis bacteria in four subgroups.

a. Sub Group1: **Photomorphogens**: These organisms grow slowly. Culture, when exposed to light turns yellow. e. g. *M. Kansasii, M. Mannum, M. Simiae*

b. Sub Group 2: **Scotochromogen:** These organisms grow slowly & form yellow pigment even in dark. When the culture is grown in light, the pigment is orange. e. g. *M. scrofulaceum, M. szulgai, M. gordonae*

c. Sub Group 3: **Nonchromogens:** Culture of these organisms is colorless & slightly pigmented; growth is slow and inhibited at 22° C but is common at 44° C. e. g *M. avium, M. intracellularae, M. ulcerans*

d. Sub group 4: **Rapid growers-** on primary culture growth may be slow, but in subcultures, growth may be evident in 2-3 days. Main organism in this sub group or category is *M. fortuitum, M. chelonae.*

2.4.3 Resistance

a. *Mycobacteria* can be killed at 60° C for 15-20 min.
b. Culture may be killed on direct sun light up to 2 hrs.
c. Bacilli may remain viable in droplet nuclei for 8-10 days
d. Bacilli in sputum may remain active for 20-30 min.
e. Culture may remain viable at Room temprature for 6-8 months and at -20 °C for 2 years.

2.4.4 Resistance to chemical disinfectants

a. Phenol 5%
b. Sulfuric acid 15%
c. Nitric acid 3%
d. Oxalic acid 5%
e. Sodium hypo chloride 4%

For 5 minutes, the organism can be destroyed by tincture of Iodine and 80% Ethanol in 2-10 min. However 80% ethanol is recommended.

2.4.5 Primary risk factor

a. Poverty
b. Malnutrition
c. Over crowding condition (Over population)
d. TB spreads more easily among family members living in the same house and breathing the same air.
e. It's spread by aerosol droplets and causes irreversible lung destruction. If it escapes from the lung, it may cause systemic disease affecting many organs including bones, joints, liver, spleen, gastrointestinal tract and brain.

2.5 Epidemiology

Progress towards eradication of bovine tuberculosis in several countries has been impeded by a lack of precise understanding of epidemiological factors, such as the significance of inter-bovine transmission and the role of wild animals in maintaining disease, as well as other husbandry and environmental factors. Skuce and Neill (2003) reported that advances in strain genotyping methods now provide the potential to assist in identifying more accurately the sources of infection and major routes of transmission as well as outbreak contacts. Systematic investigation involving strain genotyping with appropriate interventions could probably elucidate more precisely the spread of bovine tuberculosis by identifying strain movement, to and/or from contiguous herds, spread within-herd, persistence on farm, recrudescence and re-introduction. In UK and Ireland, investigating further the geographical association between badger and cattle strains could accurately validate perceived associations

between badgers, other wildlife and tuberculous cattle. The direction of *M. bovis* transmission between wildlife and cattle may be inferred from temporal and geographically based information on prevalent strains involved. Additionally, finding of unusual *M. bovis* genotypes amongst prevalent strains in a particular area with unexpectedly identified reactor cattle could alert to fraudulent activity in cattle movement. Furthermore, *M. bovis* strains may differ in their immunogenicity and pathogenicity, both of which could affect tuberculosis detection. There is already some evidence that *M. bovis* strain type may influence skin test effectiveness (Goodchild *et al.,* 2003).

Established strain typing techniques such as restriction enzyme analysis (REA), (Collins, 1999) and restriction fragment length polymorphism (RFLP) typing (Van Soolingen, 2001) have been standardized and are routine procedures in several public health and veterinary research laboratories. However, these techniques are technically demanding and require expensive softwares for analysis and archiving of complex banding patterns. The achievement of inter-laboratory reproducibility is not trivial (Heersma *et al.* 1998). Some genetic markers may lack discrimination with some members of the *M. tuberculosis* complex. For example, IS*6110*-RFLP analysis and spoligotyping (Kamerbeek *et al.,* 1997) are poorly discriminating for most *M. bovis* isolates, although spoligotyping does provide additional phylogenetic data.

In New Zealand, REA typing has provided a new insight into disease dynamics, demonstrating clustering of REA types in defined areas. Possums, other wildlife and farmed animals in the same area were often infected with

the same REA type. REA typing has been used to include or exclude possible sources of infection in specific herds and it has demonstrated clearly whether infection in farmed animals has come from the infected local wildlife reservoir, or from infected cattle and deer, brought onto those premises. REA strain typing has been used to influence the level of herd testing or wildlife control in specific areas and is now considered an integral component in local TB control schemes (Collins, 1999).

The genome sequencing projects have disclosed several different classes of genetic marker, including insertion sequences, insertion/deletion events, tandem repeat loci and point mutations (single nucleotide polymorphisms), all of which are now being exploited in molecular epidemiology studies, in an attempt to develop more convenient strain typing techniques for *M. bovis*, that are amenable to uncomplicated analysis, intuitive nomenclature and reliable inter-laboratory comparison. Identification of repetitive DNA, such as variable number tandem repeats (VNTRs) in the genome sequences of *M. tuberculosis* strains $H_{37}Rv$ and CDC 1551 (Cole *et al.,* 1998) and *M. bovis* AF2122/97 has been exploited recently in strain typing and high throughput, highly discriminating and reproducible assays have been configured (Roring *et al.,* 2004). For local applications, it is important to characterize prevalent strains and the choice and combination of VNTR markers, which are most informative in a given location, needs to be determined empirically.

A striking feature of applying both spoligotyping and the higher-resolution VNTR assay to surveillance, at least in the UK and Ireland, is that *M. bovis* strain appear to cluster in defined geographical areas (Smith *et al.,* 2003)

suggesting that sources of infection are stable and local. Combining spatial data with hypothetical phylogenies based on spoligotyping and VNTRs have suggested a series of 'clonal expansions', where a particular VNTR genotype increases dramatically in frequency. This is either via selection or ecological opportunity but most likely due to simple invasion, rather than resulting from enhanced adaptation to wildlife and / or cattle. These techniques have also been used to establish, from cattle movement data, the probability of infection being bought-in or acquired on arrival (Durr *et al.,* 2004). Strain typing has already provided evidence of within-herd spread and the introduction of infection through long-range cattle movements.

2.6 Cell structure and Metabolism

Mycobacteria are rod-shaped, Gram-positive aerobes, or facultative anaerobes. As deduced from its genome, *M. tuberculosis* has the potential to manufacture all of the machinery necessary to synthesize all of its essential vitamins, amino acids, and enzyme co-factors. On the other hand, the inability to culture *Mycobacterium leprae,* suggests that it has lost many of its metabolic capabilities, and is now an obligate parasite, dependent on its host for most of its nutritional needs (Cole *et al.,* 1998). This goes in accordance with its severely degenerated genome additionally *M. tuberculosis* has an unusual cell wall, with an additional layer beyond the peptidoglycan layer, which is rich in unusual lipids, glycolipids, and polysaccharides.

2.7 Taxonomic Status

The generic name *Mycobacterium* (Fungus - Bacterium) was first coined by Lehmann & Newmann (1996) in the 1st edition of their book Atlas of *Bacteriology*, because of the mould like pellicular growth of the tubercle bacillus on liquid media. The variations of properties within the genus *Mycobacterium* is enormous and is reflected in the range of virulence, habitat, rate of growth, nutritional requirements and antigenicity.

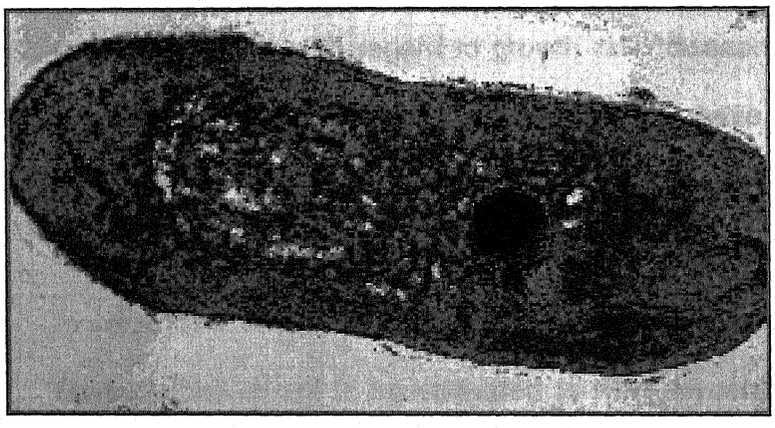


Figure 3: Electron micrograph of *M.tuberculosis.* Courtesy of the Institut Pasteur image library.

Many of the unique characteristics of *Mycobacteria* are found to be in their very complex lipid rich cell walls. *Mycobacteria* are gram-positive, aerobic, non-sporing & non-motile (Figure-3). These do not form capsules but have the lipids termed mycosides. *Mycobacteria* are important group of organisms, because many of them are pathogenic to Human as well as animals. *Mycobacteria* are gram positive, usually acid and alcohol fast at some stage of growth, they do not form capsules, endospores or conidia, rarely exhibit grossly visible aerial hyphae, produce acid from sugar by oxidation & with exception of those that do not grow *In vitro* can be divided into rapid and slow growing strain. The original description of the genus was based mainly on morphological & staining properties - features that are considered sufficiently

diagnostic to distinguish *Mycobacteria* from Actinomycetes (Skerman *et al.*, 1980).

Further publications of newly recognized species, primarily of rapid growers have now brought the number to 54.Of these 54, 17 species are pathogenic & others are non-pathogenic. Members of genus *Mycobacterium* exhibit a great deal of variability with regard to growth rate, disease causing capability and physiology. Different *Mycobacterial* species have been described and classified on the basis of rate of growth, chromogenecity etc. Although *Mycobacteria* were not formerly divided into subgenera, it is almost essential to consider it in two major categories. With the exception of *Mycobacterium leprae*, which is still to be cultivated *in vitro*, *Mycobacteria* can be assigned into two groups primarily on the basis of growth rates. The so called rapid growers include strains of those species which under optimal conditions of nutrition & temperature produce colonies within seven days, whereas, their slow growing counterparts require an additional view or more to form such colonies under such conditions. The optimum temperature of both species ranges from 28- 45 °C.

2.8 Mycobacterial genome

M. tuberculosis $H_{37}Rv$ genome comprises of 4,411,529 base pairs, and contains around 4000 genes (3924 predicted protein coding genes). The genome has a very high guanine + cytosine content (65.6 %) that is reflected in the biased amino acid content of the proteins. There is a significant

preference for the amino acids Ala, Gly, Pro, Arg and Trp, which are all encoded by G+C rich codons, and a comparative reduction in the use of amino acids encoded by A+ T rich codons such as Asn, Ile, Lys, Phe and Tyr. *M. tuberculosis* radically differs from other bacteria in a very large portion of its coding capacity is devoted to the enzymes involved in lipogenesis and lipolysis. In total there are ~250 distinct enzymes involved in fatty acid metabolism in *M. tuberculosis* compared with only 50 in *E. coli* (Cole *et al.,* 1998).

2.9 TRANSMISSION

The principal risk for acquiring infection with *Mycobacterium tuberculosis* is breathing (Bloom and Murray 1992). The classic studies of Wells (1955) and his student Riley (Riley and O'Grady, 1961) established beyond doubt that infectious particles containing *M. tuberculosis* were emitted in coughs and sneezes and even while speaking, a sneeze may contain over a million particles with diameters of less than 100μm, the mean being 10 μm. This particle from droplet nuclei in which evaporation continues until the vapor pressure of the droplet equals the atmospheric pressure. The droplet nucleus is very stable, settles very slowly (about 12mm/min), and remains suspended in the air for long periods, A 10 μm droplet nucleus may carry perhaps 3 to 10 tubercle bacilli. Dust associated particles may also carry *M. tuberculosis.* These particles are larger than droplets, but they can be transiently resuspended by air convection and may serve as a reservoir for infectious bacilli. Infection can take place by ingestion of tubercle bacilli but is about 10,000 fold less effective than inhalation of droplets on transmitting

tuberculosis; probably tubercle bacilli are very sensitive to gastric acid (Gaudier and Gernez-Rieux, 1962). The number and concentration of bacilli present on the source case estimated to be between 10^2 to 10^3 in solid or nodular lesions but of the order of 10^2 to 10^3 in cavitary lesions (Canetti, 1965), are major variables in transmission, duration of exposure and the aerodynamics of the exhaled particles.

Thus lung is major portal of entry in the majority of cases of tuberculosis (Glassroth *et al.,* 1980s; Mayock and Mac, 1976). Pulmonary TB has been variously described as consumption. The terms indicating the severe wasting and the coughing of blood associated with later stages of the disease. Tuberculosis also can develop in the central nervous system, in which case meningitis is the predominant from of the disease and also in the urogenital tract, the digestive system and cutaneously in the from named lupus vulgaris. The incidence of these various extrapulmonary forms of tuberculosis varies from country to country, such that on the average between 1964 and 1989, 20 percent of the 20,000 new cases of TB in the United States were extrapulmonary while 5 to 10 percent of the approximately seven million new cases each year in the developing countries were extrapulmonary (Talavera *et al.,* 2001).

This distribution also can be affected by origin of the individuals within a country. In one study of TB patients in England, 20 percent of patients of European origin had extrapulmonary TB, of which lymph node, bone and joint and genitourinary involvement accounted for almost 90 percent. Of patients whose origin was on the Indian subcontinent, 45 percent had extrapulmonary

tuberculosis and 60 percent of these sites of infection were in lymph nodes and in bones and joints (Yates, and Grange, 1993). Autopsies of deceased human immunodeficiency virus (HIV) negative TB patient in another study in New York City showed that 68 percent had extrapulmonary TB whose lesions were widely and randomly distributed throughout the body with no apparent predilection for a limited number of sites as noted in the English study (Jagirdar and ZagZag, 1996).

Tuberculosis usually affects the lungs although in up to one-third of cases, other organs are involved. In order of frequency, the extrapulmonary sites most commonly involved in tuberculosis are the lymph nodes, pleura, genitourinary tract, bones and joints, meningitis and peritoneum. However, virtually all organ systems may be affected as a result of hematogenous dissemination in HIV-infected individuals, extrapulmonary tuberculosis is seen more commonly today than in the past.

The risk of developing disease after being infected depends largely on endogenous factors, such as the individual's innate susceptibility to disease and level of function of cell-mediated immunity.

Age is an important determination of the risk of disease after infection. Among infected persons, the incidence of tuberculosis is highest during late adolescence and early adulthood, the reasons are unclear.

The incidence among women peak at 25 to 34 years of age group rates among women are usually higher than those among men, while at older ages the opposite is true. The risk may increase in the elderly, possibly because of waning immunity and co morbidity.

A variety of diseases favor the development of active tuberculosis. The most potent risk factor for tuberculosis among infected individuals is clearly HIV co-infection, which suppresses cellular immunity. The risk that latent *M. tuberculosis* infection will precede to active disease is directly related to the patient's degree of Immunosuppression. In a study of HIV-infected, PPD-positive person, this risk varied from 2.6 to 13.3 cases per 100 person –years and depend upon the $CD4^+$ cell count. This risk of developing tuberculosis is significantly greater among HIV-infected than among HIV- uninfected hosts. Other conditions known to increase the risk of active tuberculosis among person infected with tubercle bacilli include silicosis, lymphoma, leukemia and other malignant neoplasm's, hemophilia, chronic renal failure and hemodialysis, insulin-dependent diabetes mellitus, immunosuppressive treatment, including that administered for solid-organ transplantation and condition associated with malnutrition such as gastrectomy and jejunoileal byepass surgery. Finally, the presence of old self-healed fibrotic tuberculosis lesion constitutes a serious risk of active disease. (Handbook of Harrison's, 2001).

In modern times, the respiratory route of exposure initiates most TB infections now that milk products are generally TB cases were pulmonary (Hopewell, 1994). Thus, the different forms of the disease discussed above usually arise from dissemination of the bacilli from infected lungs. TB in many cases follows

a general pattern as described by Wallgren, who divided the progression and resolution of the disease into four stages (Wallgren, 1948). In the first stage, dating from 3 to 8 weeks after *M. tuberculosis* contained in inhaled aerosols becomes implanted in alveoli the bacteria are disseminated by the lymphatic circulation to regional lymph nodes in the lung, forming the so-called primary or Ghon complex. At this time, conversion to tuberculin reactivity occurs. The second stage, lasting about 3 months, is marked by hematogenous circulation of bacteria to many organs including other parts of the lung; at this time in some individuals, acute and sometimes fatal disease can occur in the form of tuberculosis meningitis (disseminated) tuberculosis. Pleurisy or inflammation of the pleural surface can occur during the third stage, lasting 3 to 7 months and causing severe chest pain, but this stage can be delayed for up to 2 years. It is thought that this condition is caused by either hematogenous dissemination or the release of bacteria into the pleural space from sub pleural concentrations of bacteria in the lung. The free bacteria or their components are thought to interact with sensitized CD4 T-lymphocytes that are attracted and then proliferate and release inflammatory cytokines (Kamholz, 1996). The last stage or resolution of the primary complex, where the disease does not progress, may take up to 3 years. In this stage, more slowly developing extrapulmonary lesions, e.g., those in bones and joints, frequently presenting as chronic back pain, can appear in some individuals. However, most humans who are infected with TB do not exhibit progression of the disease.

One-third of exposed HIV-negative individuals become infected, and of this number 3 to 5% develop TB in the first year. An additional 3 to 5% of those infected develop TB later in their lives. It is thought that most adult TB in non-HIV-infected patients is caused by reactivation of preexisting infection (Garay, 1996). HIV-positive persons infected with *M. tuberculosis* have a 50% chance of developing reactivation (post primary) TB at some time in their lives. These individuals and others who are immunosuppressed can also be newly infected with *M. tuberculosis* and in many cases show rapid progression to active disease (Garay, 1996 b). Adult TB, whether resulting from activation or new infection in HIV-infected patients, is almost always pulmonary and is associated with differing degrees of lung involvement and damage, notably necrosis, cavitation and bleeding (Jagirdar and ZagZag, 1996).

As discussed above, the interaction of *M. tuberculosis* with the human host begins when droplet nuclei containing microorganisms from infectious patients are inhaled. While the majority of inhaled bacilli are trapped in the upper airways and expelled by ciliated mucosal cells, a fraction (usually fewer than 10%) reaches the alveoli. It is also possible that bacteria can be initially ingested by epithelial type-II pneumocytes. This cell type is found in greater numbers than macrophages in alveoli and *M. tuberculosis* can infect and grow in these pneumocytes *ex vivo* (Bermudez and Goodman, 1996; Mehta *et al.*, 1996). In addition, dendritic cells play a very important role in the early stages of infection since they are much better antigen presenters than are macrophages and presumably play a key role in activating T-cells with specific *M. tuberculosis* antigens (Bodnar *et al.*, 2001; Gonzalez-Juarrero *et*

al., 2001). Since dendritic cells are of migrating nature unlike differentiated macrophages (Lipscomb and Masten, 2002), they also may play an important role in dissemination of *M. tuberculosis.* Invasion of macrophages by *Mycobacteria* may result in part from association of C2a with the bacterial cell wall followed by C3b opsonization of the bacteria and recognition by the macrophages. The bacteria are phagocytosed in a process that is initiated by bacterial contact with macrophage mannose and / or complement receptors (Schlesinger, 1993). Surfactant protein A, a glycoprotein found on alveolar surfaces can enhance the binding and uptake of *M. tuberculosis* by up regulating mannose receptor activity (Gaynor *et al.,* 1995).

On the other hand, surfactant protein D, similarly located in alveoli, inhibits phagocytosis of *M tuberculosis* by blocking mannosyl oligosaccharide residues on the bacterial cell surface (Ferguson *et al.,* 1999), and it is proposed that this prevents *M. tuberculosis* interaction with mannose receptors on the macrophage cell surface. Cholesterol in cell plasma membranes is thought to be important for this process, since removal of this steroid from human neutrophils decreases the phagocytosis of *M. kansasii* (Peyron *et al.,* 2000) and similar depletion experiments prevented the entry of *M. bovis* BCG into mouse macrophages (Gatfield and Pieters, 2000). The human toll-like receptor 2 (TLR2) also plays a role in *M. tuberculosis* uptake (Noss *et al.,* 2001).

On entry into a host macrophage, *M. tuberculosis* and other intracellular pathogens initially reside in an endocytic vacuole called the phagosome. If the normal phagosomal maturation cycle occurs, i. e., phagosome-lysosome

fusion, these bacteria can encounter a hostile environment that includes acidic pH, reactive oxygen intermediates (ROI), lysosomal enzymes, and toxic peptides. Reactive nitrogen intermediates (RNIs) produced by activated mouse macrophages are major elements in antimicrobial activity (Nathan and Hibbs, 1991), and mice with mutations in the genes encoding the macrophage-localized cytokine inducible nitric oxide synthase genes are more susceptible to various pathogens, including *Leishmania major* (Wei, 1995), *Listeria monocytogenes* (MacMicking, 1995), and *M. tuberculosis* (MacMicking, 1997). The *M. tuberculosis* result is consistent with the results of other experiments showing that RNIs are the most significant weapon against virulent *Mycobacteria* in mouse macrophages (Chan *et al.,* 1995; Chan *et al.,* 1992) and the observation that resistance to RNIs among various strains of *M. tuberculosis* correlates with virulence (Chan *et al.,* 1995; Chan *et al.,* 1992; O'Brien *et al.,* 1994). The presence of RNIs in human macrophages and their potential role in disease has been the subject of controversy, but the alveolar macrophages of majority of TB-infected patients exhibit iNOS activity (Nicholson *et al.,* 1996).

2.10 Survival of *M. tuberculosis*

Accumulation evidence suggests that *M. tuberculosis* enters macrophages via specific binding to cell surface molecule of phagocytes. It has been reported that the tubercle bacillus can bind directly to the mannose receptor via the cell wall-associated, mannosylated glycolipid LAM or indirectly via complement receptors of the integrin family (CR1, CR3) or Fc receptors. Phagocytosis, triggered by engaging certain cell surface molecules such as the Fc receptor,

stimulates the production of ROI via activation of the oxidative burst. Experimental data indicate that *M. tuberculosis* can interfere with the toxic effect of ROI by various mechanisms. First, various *Mycobacterial* compounds including glycolipids (GL), sulfatides (ST) and LAM can down regulate the oxidative cytotoxic mechanism. Second uptake via CR1 bypasses activation of the respiratory burst. Cytokine-activated macrophages produce RNI that, at least in the mouse system, mediate potent antiMycobacterial activity. The acidic condition of the phagolysomal vacuole can be conductive to the toxic effect of RNI. However, NH_4^+ production by *M. tuberculosis* may attenuate the potency of the L-arginine-dependent antiMycobacterial mechanism and that of lysosomal enzymes, which operate best at an acidic pH. In addition, *Mycobacterial* products such as sulfatides and NH_4^+ may interfere with phagolysosomal fusion. Finally, the tubercle bacillus may evade the highly toxic environment by escaping in to the cytoplasm via the production of hemolysin.

2.11 Pathogenesis & Immunology

Pathogenicity for human for some of the following bacteria is questionable: *M. gordonae, M. asisticum, M. terrae, M. triviale, M. nonchromogenicum, M. gastri, M. flavescens* and *M. phlei.* These species may be found in sputum specimens, excent for *M. marinum, M. haemophilum and M. ulcerans*, which are typical of specimens from skin lesions. Any of these species, can be found in blood specimens obtained from HIV-infected individuals, although *M. avium* is most often isolated. Since *M. tuberculosis* can also be isolated from blood specimens, one must give special attention to the possibility of mixed culture.

The frequency of finding more than one *Mycobacterial* species in sputum specimens from non-HIV patients is increasing as well.

2.11.1 Pulmonary TB

The most common clinical manifestation of TB is the pulmonary disease. After inhalation, the bacilli initiate small lesions in the lower respiratory tract. These lesions frequently heal to form tiny tubercle, which are too small to be seen by x-rays but may continue to harbor the bacilli indefinitely. In other cases, replication of the bacilli continues and the lesions expand and undergo caseation necrosis, which will destroy the normal tissue and leave the necrotic tissue in a semisolid, "cheesy" state. Caseation necrosis may eventually heal and become infiltrated with fibrous tissue and calcium deposits, or may continue to expand leaving cavities in the lungs (Gebbardt *et al.,* 1996).

3.11.2 Extra pulmonary TB

Extrapulmonary TB is more common in children and in HIV-infected individuals (Shafer and Edlin, 1996). In extrapulmonary TB, the tubercle bacilli may spread through the bloodstream from the lesions in the lungs into other organs such as, bones and joints particularly the spine (Okuyama *et al.,* 1996), kidneys and genital tract causing genitourinary TB (Gorse and Belshe, 1985), or the central nervous system causing TB meningitis (Thwaites *et al.,* 2000). TB meningitis is fatal in almost all cases without treatment, therefore prompt identification and chemotherapy are crucial to prevent serious neurological sequels.

Another clinical manifestation of extrapulmonary TB is disseminated TB, which is defined as involvement of many organs simultaneously, and can occur as result of a primary progressive disease or reactivation of the latent infection (Hill *et al.,* 1991).

The pathogenesis of tuberculosis described series of battles between the host and the parasite; the weapons of the host are - 1) activated macrophage- to kill the tubercle bacilli that it ingests. 2) Inhibitory- potential to stop the intracellular growth of bacilli in a non-activated macrophage by killing the macrophage, thereby transforming a favorable intracellular environment in to the inhibitory environment of solid caseous tissue.

The weapons of tubercle bacillus are (1) Logarithmic multiplication ability within non-activated macrophages, i. e., within the monocytes that recently immigrated into the tissues' local sites of the infections. (2) Extracellular multiplication ability, often reaching tremendous numbers, in the liquefied caseous focus which often forms activity.

Despite the strong host weapons against tubercle bacillus the host is vulnerable due to (1) the non-activated macrophages, which provide a favorable intracellular environment are bacillary growth and (2) material which is the only menstruum that supports the extracellular growth of the bacillus.
The vulnerability of the bacillus are- (1) an inability to survive within a fully activated macrophage. (2) An inability to multiply in solid caseous tissue.
Cell mediated immunity (CMI), aquired cellular resistance (ACR), and

Delayed-type hypersensitivity (DTH) are immunolgical responses produced by the host that play key role in the pathogenesis of tuberculosis. The tubercle bacillus apparently cannot injure host tissue until these immune responses develop.

Usually the control of the infection at some point during various stages of disease progression in the sensitive host, the following stages have been marked- strain of M.TB prior exposure, vaccination, Infectious dose, Immune status of the host.

2.11.2 Stages of Tuberculosis infection

Stage-1.

There is no bacillary growth. The bacillus growth, the bacillus is usually destroyed or inhibited by the mature resident AM that ingests it. If the bacillus is not destroyed, it grows (i. e. multiplies) and eventually destroys the AM.

Stage-2.

This begins after 7-21 days of initial infection. *Mycobacterium* Multiples logarithmically with the immature unactivated macrophages of the developing lesion (now called a tubercle). This ultimately leafs to burst of the macrophages. Other macrophages begin to extravasate from peripheral blood. These macrophages also phagocytose M. TB, but they are also inactivated and hence cannot destroy M. TB.

Stage-3.

The number of viable bacilli becomes stationary because there growth is inhibited by the immune response. This immune response has two components- (a) Cell-mediated immunity (CMI), i. e., activated macrophages

and (b) delayed-type hypersensitivity (DTH), i. e. caseous necrosis. Cell-mediated immunity is critical at this early stage which confers partial protection against *M. tuberculosis*, while humoral immunity has no defined role in protection. Two types of cells are essential macrophages, which directly phagocytize tubercle bacilli and T-cells (mainly $CD4^+$ lymphocytes), which induce protection through the protection of lymphokines. At this stage lymphocytes begin to infiltrate. The lymphocytes, specifically T-cells, recognize processed and presented M. TB antigen in content of molecule. Coincident with the appearance of immunity, DTH to *M. tuberculosis* develops. This reactivity is the basis of the PPD skin test, currently the only test that reliably detects *M. tuberculosis* infection in persons without symptoms. The cellular mechanisms responsible for PPD reactivity are related mainly to previously sensitized $CD4^+$ lymphocytes, which are attracted to the skin-test site. There, they proliferate and produce cytokines.

Antigen processing and presentation by macrophages to T-lymphocytes result in proliferation of $CD4^+$ lymphocytes, which are crucial to the host's defense against *M. tuberculosis.* Qualitative and quantitative defects of $CD4^+$ T cells explain the inability of HIV-infected individuals to contain *Mycobacterial* proliferation. Reactive $CD4^+$ lymphocytes produce cytokines of the T_H1 pattern and participate in MHC class II-restricted killing of cells infected with *M. tuberculosis.* T_H1 $CD4^+$ cells produce interferon- α (IFN- α) and IL-2 and promote cell-mediated immunity. T_H2 cells produce IL-4, IL-5, and IL-10 and promote humoral immunity. The interplay of these various cytokines and their cross-regulation determine the host's response. The role of cytokines in

promoting intracellular killing of *Mycobacteria* has not been entirely elucidated. IFN-α may induce release of nitric oxide, and TNF-α also seems to be important. Finally, the role of other cells, such as natural killer (NK) cells, "double-negative" $CD4^+$, $CD8^+$ cells, and T cells, in protective immunity remains unclear.

It is at this stage that the individual becomes tuberculin-positive. This positive tuberculin reaction is the result of the host developing a vigorous cell mediated immune (CMI) response. A CMI response must be mounted to control an M. TB infection. An antibody-mediated immunity (AMI) will not aid in the control of a M. TB. Infection because M. TB is intracellular and if extracellular, it is resistant to complement killing due to the high lipid concentration in its cell wall.

Although, CMI response is necessary to control *M. tuberculosis* infection. It is also responsible for much of the pathology associated with tuberculosis. Activated macrophages may release lytic enzymes and reactive intermediates that facilitate the development of immune pathology. Activated macrophages and T-cells also secrete cytokines that may also play a role in the development of immune pathology, including Interleukin 1 (IL-l), tumor necrosis factor (TNF), and gamma IFN, contributes to the killing of *Mycobacteria*, the formation of granulomas and a number of systemic effects such as fever and weight loss. It is at this stage that tubercle formation begins. The center of the tubercle is characterized by 'caseation necrosis' meaning semi-solid or 'cheesy' consistency M. TB. Cannot multiply within

these tubercles because of the low pH and anoxic environment. M. TB can however, persist within these tubercles for extended periods. The disease is arrested at this stage if the activated macrophage population predominates.

In next stages many activated macrophages can be found surrounding the tubercles, many other macrophages present remain unactivated or poorly activated. M. TB uses these macrophages to replicate and hence the tubercle grows. The growing tubercle may invade a bronchus. If this happens, M.TB infection can spread to other parts of the lung. Similarly the tubercle may invade an artery or other blood supply line. The hematogenous spread of M.TB may result in extrapulmonary tuberculosis otherwise known as milliary tuberculosis. The name "milliary" is derived from the fact that metastasizing tubercles are about the same size as a millet seed, a grain commonly grown in Africa.

The secondary lesions caused by milliary TB can occur at almost any anatomical location, but usually involve the genitourinary system, bones, joints, lymph nodes, and peritoneum. These lesions are of two types:

1.Exudative lesions result from the accumulation of PMN's around M. TB. Here the bacteria replicate with virtually no resistance. This situation gives rise to the formation of a "soft tubercle".

2.Productive or granulomatous lesions occur when the host becomes hypersensitive to tuberculoproteins. This situation gives rise to the formation of a "hard tubercle".

In the last stage of the liquefaction the bacillus evades host defenses. Reasons leading to liquefaction of caseous centers of tuberculosis remains are still unknown. This liquid is very conducive to M.TB growth and hence the organism begins to rapidly multiply extracellularly. After some time, the large antigen load causes the walls of nearby bronchi to become necrotic and rupture. This result in cavity formation, the activity due to the tissue destruction as a result of a delayed-type hypersensitivity (DTH) reaction to various bacillary antigens. The bacilli enter the bronchial tree and then other parts of the lung and the outside environment. Arrestment of the disease at this stage depends on whether the antigenic load (off both the bacilli and their products) remains small enough for the host to cope with only a very small percent of M.TB infections resulting in the formation of disease and even a smaller percentage of M. TB infection progress to an advanced stage. Usually the host will begin to control the infection at some point. When the primary lesion heals, it becomes fibrous and calcifies. When this happens, the lesion is referred to as the Ghon complex. Depending on the size and severity, the Ghon complex may never subside. Typically the Ghon complex is readily visible upon chest X-ray.

2.11.2.1 Immune response to TB

Innate immune response

Recent immunological and genetic studies have corroborated the long-standing notion that, innate immunity is relevant in the host defense against *M. tuberculosis.*

The uptake of *M. tuberculosis* by alveolar macrophages represents the first step in the innate host defense against TB. This initial interaction is mediated by cellular receptors such as complement receptors, mannose receptors, surfactant receptors, and scavenger receptors (Chan *et al.,* 1992; Downing *et al.,* 1995; Flesch and Kaufmann, 1988; Gaynor *et al.,* 1995; Schlesinger *et al.,* 1993). Most recently, attention has been focused on the role of toll-like receptors (TLRs) in mediating the uptake of *Mycobacteria* by macrophages.

Specifically, the role of TLR2 and TLR4 in sensing *Mycobacteria* and promoting anti-*Mycobacterial* responses has been demonstrated in several studies. *In vivo* studies using TLR2 or TLR4 deficient mice have shown that these mice are more susceptible to *Mycobacteria* infection than wild-type mice (Quesniaux *et al.,* 2004).

Furthermore, *in vitro* studies using a human macrophage-like cell line have demonstrated that activation of TLRs by lipoproteins contained within the *M. tuberculosis* cell wall induces production of IL-12, an important pro-inflammatory cytokine in the host response against TB (Brightbill *et al.,* 1999). In addition, these studies showed that TLR-mediated IL-12 production also resulted in increased production of nitric oxide synthetase and nitric oxide, which are important for the intracellular killing of *Mycobacteria.*

Thus, TLRs contribute to the innate immunity by detecting *Mycobacteria*-associated molecular patterns and mediating the secretion of antiMycobacterial effector molecules. However, TLRs can also influence the

specific immunity by upregulation of immunomodulatory molecules supporting the development of pro-inflammatory responses (Schluger, 2001).

2.11.2.2 Specific immune response

The specific immune response to *M. tuberculosis* in the lungs is complex and involves multiple mechanisms. T cells are believed to be essential in the protective immune response against TB, and the interaction of T cells with macrophages is critical for the control of the infection. The production of inflammatory cytokines and chemokines, induced by ingestion of *M. tuberculosis* by alveolar macrophages (Means *et al.,* 1999), leads to the migration of monocyte-derived macrophages and dendritic cells to the site of infection. The dendritic cells that engulf *Mycobacteria* mature and migrate to regional lymph nodes (Bodnar *et al.,* 2001; Henderson *et al.,* 1997; Hertz *et al.,* 2001), where then T cells are primed against *Mycobacteria* antigens. Primed T cells expand and migrate to the site of infection in the lungs, presumably due to the upregulation of local adhesion molecules and chemokines. The migration of macrophages and T cells to the site of infection results in formation of a granuloma (Figure- 4), which also comprises other cells such as B cells, dendritic cells, endothelial cells, fibroblasts and probably stromal cells (Gonzalez-Juarrero *et al.,* 2001).

The granuloma functions as an immune microenvironment to facilitate interactions between T cells and macrophages. In addition to providing a framework for these cells, granulomas serve to wall off *Mycobacteria* from the rest of the lungs, limiting the dissemination of the infection. However,

depending on the cellular composition and on the cytokine and chemokine-secreting profile, granulomas can also be associated with pathology or at least lack of adequate containment of bacillary multiplication (Saunders and Cooper, 2000).

2.11.3 Role of CD4, CD8 and γδ T cells in the response against *M. tuberculosis* and latent infection

2.11.3.1: $CD4^+$ T cells

CD4+ T cells play a central role in the immune response against *M. tuberculosis*. Peptide antigens from *Mycobacteria,* degraded in the phagolysosomal compartment and complexed with the MHC class II molecules are recognized by $CD4^+$ T cells, resulting in their activation (Davis and Bjorkman, 1988). The main function of $CD4^+$ T cells in immunity to TB is thought to be the production of cytokines, specifically IFN-γ, which is critical for macrophage activation and the subsequent induction of microbicidal mechanisms (Flesch and Kaufmann, 1990). The critical role of IFN-γ in the control of *Mycobacteria* infections has been demonstrated in animal models. Experimentally, mice deficient in IFN-γ or in IL-12, a critical cytokine in the induction of IFN-γ production, were highly susceptible to challenge with *M. tuberculosis* (Cooper *et al.,* 1993; Cooper *et al.,* 1997). In addition, studies in humans have shown that patients with IFN-γ receptor deficiency presented disseminated infection with *M. bovis* BCG and/or environmental *Mycobacteria*, which resulted in the death of about half of the patients and required continuous anti-*Mycobacterial* treatment in the survivors (Abel *et al.,* 2002).

$CD4^+$ T cells can also contribute to the control of acute *Mycobacteria* infections through IFN-γ independent mechanisms. This has been

demonstrated in a variety of experimental models using antibody depletion and mouse strains deficient in either CD4 or MHC class II molecules (Caruso *et al.,* 1999; Scanga *et al.,* 2000). In mice deficient in CD4 or MHC class II molecules, the levels of IFN-γ were significantly diminished very early during infection, but later the IFN-γ production was similar to that seen in wild type mice. However, deficient mice were not rescued by this later production of IFN-γ, and succumbed to the infection. IFN-γ independent mechanisms of action of $CD4^+$ T cells may also include a cytolytic function of these cells, as has been shown in murine models (Izzo and North, 1992) as well as in humans (Tan *et al.*, 1997).

Further evidence of the importance of $CD4^+$ T cells in the control of TB in humans is obtained from studies of the clinical course of co-infection with HIV. Depletion of $CD4^+$ T cells during HIV infection dramatically increases the susceptibility to primary and reactivation TB (Havlir and Barnes, 1999; Jones *et al.,* 1993).

2.11.3.2 $CD8^+$ T cells

Despite the intraphagosomal location of *M. tuberculosis*, it is now recognized that $CD8^+$ T cells, restricted either by MHC class I or CD1 molecules, participate in a successful anti-*Mycobacterial* immune response. In contrast to the peptide epitopes presented by the MHC molecules, CD1 molecules present lipids or glycolipids to T cells (Porcelli and Modlin, 1999).

Experimentally, mice deficient in β2-microglobulin, a component of both MHC class I and non-classical MHC class 1b molecules were found to be more susceptible to infection with *M. tuberculosis* than wild type mice (Flynn *et al.*, 1992). Similarly, increased susceptibility to *Mycobacterial* infections has been seen in mice deficient in transporters associated with antigen processing (TAP) molecules, which transport peptides from the cytosol to the endoplasmic reticulum for loading into MHC class I molecules (Behar *et al.*, 1999; Sousa *et al.*, 2000). In addition to these studies, vaccination of mice with DNA plasmids expressing *Mycobacterial* antigens were also shown to induce antigen-specific $CD8^+$ CTL, which conferred protection against challenge with *M. tuberculosis* (Smith and Dockrell, 2000). Despite all the experimental findings confirming the role of $CD8^+$ T cells in the control of TB, it still remains unclear how phagosomically derived antigens interact with the MHC class I processing machinery.

$CD8^+$ T cells appear to have two major functions in TB immunity, lysis of infected cells and production of cytokines, mainly IFN-γ. The relative contribution of these functions is unknown. It has been shown that $CD8^+$ T cells from the lungs of infected mice are primed to produce IFN-γ, upon T cell receptor (TCR) interaction with *M. tuberculosis* infected dendritic cells (Serbina and Flynn, 1999). However, unlike $CD4^+$ T cells spontaneous *ex vivo* production of IFN-γ by $CD8^+$ T cells is very low, suggesting that the production of this cytokine by $CD8^+$ T cells in the lungs is limited (Serbina and Flynn, 1999). Evidence for a more direct role of $CD8^+$ T cells come from studies showing lysis of infected human macrophages and dendritic cells by CD1 and

MHC class I restricted $CD8^+$ T cells specific for *M. tuberculosis*, resulting in reduced numbers of intracellular bacteria (Cho *et al.*, 2000; Stenger *et al.*, 1997). The killing of the intracellular bacteria was shown to be perforin-dependent (Stenger *et al.*, 1997). Perforin was required to form a pore, but the molecule responsible for the killing of the intracellular bacteria was granulysin, another cytotoxic granule protein (Stenger *et al.*, 1998).

2.11.3.3: $\gamma\delta^+$ T cells

A large amount of evidence from human and animal studies suggests that, γδ+ T cells play a significant role in the host response to TB (Boom, 1996). It is generally believed that these cells are involved in primary immune defense. Indeed, a recent study reported that γδ+ T cells accumulated in the lungs of BCG-infected mice three weeks earlier than antigen-specific αβ+ T cells, suggesting that γδ+ T cells in the lungs might help to control *Mycobacterial* infection during the period between the innate and adaptive immunity.

Additionally, results suggested that γδ+ T cells might also play an important regulatory role in the subsequent onset of αβ+ T cells (Dieli *et al.*, 2003).

Experimentally, expansion of γδ+ T cells has been shown in mice exposed to *Mycobacterial* antigens or live bacteria. In addition, *in vitro* studies have also shown expansion of human γδ+ T cells, specially the Vγ9/Vδ2 TCR subset, by *Mycobacterial* antigens and live *Mycobacteria* (Boom *et al.*, 1992; De Libero *et al.*, 1991; Havlir *et al.*, 1991; Ohmen *et al.*, 1991; Panchamoorthy *et al.*, 1991; Porcelli *et al.*, 1992).

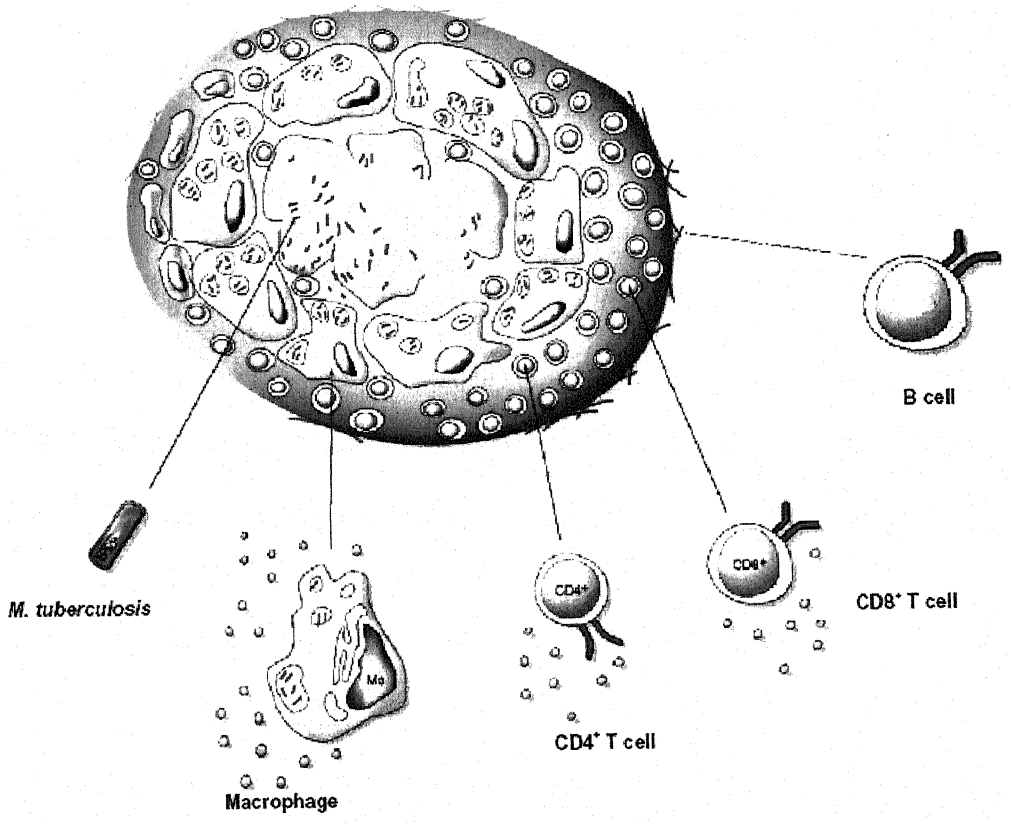


Figure 4: Structural organization of a granuloma.

M. tuberculosis reactive γδ+ T cells have been detected in the peripheral blood of tuberculin skin test (TST) positive and BCG-vaccinated individuals. These cells were found to be cytotoxic for monocytes pulsed with *Mycobacterial* antigens and to secrete cytokines that may be involved in the granuloma formation (Cooper, 1993; Munk *et al.,* 1990).

The role of γδ+ T cells in the granuloma formation in response to *M. tuberculosis* has been demonstrated in studies using mice with severe combined immunodeficiencies (SCID). In these studies, SCID mice did not form granulomas and rapidly succumbed to disease after BCG infection. However, these mice survived BCG inoculation, when engrafted with co-isogenic lymph node cells depleted of $CD4^+$ and $CD8^+$ T cells, indicating that the remaining $\gamma\delta^+$ T cells were responsible for this response (Izzo & North, 1992; North and Izzo, 1993).

2.11.4 Macrophages

Apart from their significant function in innate immunity, macrophages have been reported to play a crucial role in the adaptive immune responses against *Mycobacteria* by producing cytokines such as tumor necrosis factor-alpha (TNF-α) and IL-1β (Fenton and Vermeulen, 1996). The importance of TNF-α has been extensively studied in knock-out mice. In these studies, TNF-α knockout mouse presented a profound susceptibility to aerogenic infection with *M. tuberculosis* characterized by a reduced macrophage differentiation and granuloma formation that resulted in *Mycobacterial* overgrowth and rapid animal death (Saunders and Cooper, 2000). Additionally, TNF-α and IL-1β

along with IFN-γ produced by T cells, stimulate production of nitric oxide in macrophages. The production of nitric oxide and related reactive nitrogen intermediates by macrophages is considered to be an effective host-defense mechanism against microbial intracellular pathogens like *Mycobacteria* (Chan *et al.,* 1992; Denis, 1991). In the murine model of TB, nitric oxide plays an essential role in the killing of *M. tuberculosis* by mononuclear phagocytes. For example, in the mouse strain with a genetic disruption for inducible nitric oxide synthetase (iNOS), infection with *M. tuberculosis* is associated with a significantly higher risk of dissemination and mortality. Although more controversial in humans, there is a growing body of evidence that nitric oxide produced by TB-infected macrophages has *anti-Mycobacterial* effects against *M. tuberculosis.* The precise mechanism(s) by which nitric oxide and other reactive nitrogen species antagonize *M. tuberculosis* is not known, but may involve disruption of bacterial DNA, proteins, signaling, and/or induction of apoptosis of macrophages that harbor *Mycobacteria.*

2.11.4.1 B cells

While the role of T cells in the protection against *Mycobacterial* infections is well established, the role of B cells and antibodies is less understood. Studies conducted in mice lacking B cells have been controversial, making it difficult to define the role of these cells in anti-*Mycobacterial* immunity. In this regard, it has been reported that B cells play no role at all (Johnson *et al.,* 1997). On the other hand, other studies have suggested a role for B cells as APCs and in granuloma formation (Vordermeier *et al.,* 1996), or a role in the regulation of chemokines and/or adhesion molecules expression leading to recruitment of neutrophils, macrophages and $CD8^+$ T cells during early *M. tuberculosis*

infection. (Bosio *et al.,* 2000). Moreover, attempts at passive vaccination with antibodies in man and mice have also produced contradictory results, having reported no effect (Glatman-Freedman and Casadevall, 1998) or inhibition of bacilli dissemination (Pethe *et al.,* 2001) and prolongation of survival in infected animals (Teitelbaum, 1998).

2.12 Virulence factor

Mycobacterium does not possess classical virulence factors like those which are the major casuses of disease due to other pathogens, e. g., toxins produced by *Corynebacterium diphtheriae, Escherichia coli* 0157:H7 *Shigelia dysenteriae* and *Vibrio cholerae* and other factors like capsules and fimbriae. However, a number of structural and physiological properties of the bacterium are beginning to be recognized for their contribution to bacterial virulence and The pathology of tuberculosis.

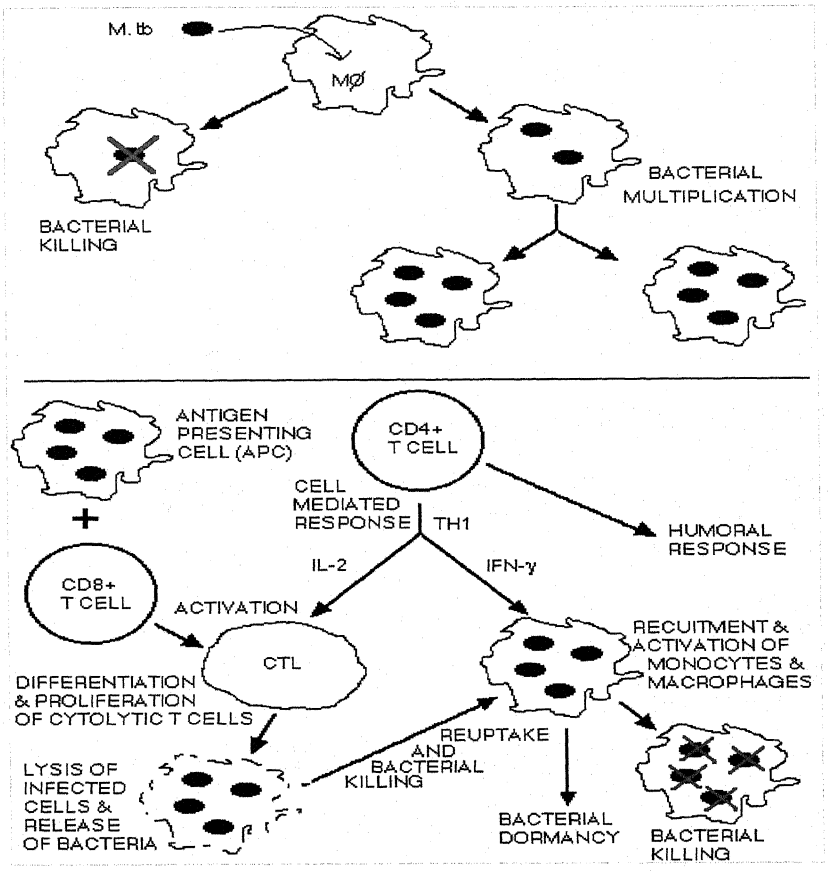


Figure 5: Flow chart showing the progression of *M. tuberculosis* bacillus after inhalation and entry into macrophages and activation of immune response.

2.13 Mechanisms for cell entry

The tubercle bacillus can bind directly to mannose receptors on macrophages via the cell wall associated mannosylated glycolipid, LAM, or indirectly via certain complement receptors or Fc receptors. On the other hand, surfactant protein D, similarly located in alveolae, inhibits phagocytosis of *M. tuberculosis* by blocking mannosyl oligosaccharide residues on the bacterial cell surface (Ferguson *et al.,* 1999), and it is proposed that this prevents *M. tuberculosis* interaction with mannose receptors on the macrophage cell surface. Cholesterol in cell plasma membranes is thought to be important for this process, since removal of this steroid from human neutrophils decreases the phagocytosis of *M. kansasii* (Piddington *et al.,* 2001) and similar depletion experiments prevented the entry of *M. bovis* BCG into mouse macrophages (Gatfield and Pieters, 2000). The human toll-like receptor 2 (TLR2) also plays a role in *M. tuberculosis* uptake (Frischkorn *et al.,* 1998).

Intracellular Growth. This is an effective means of evading the immune system. In particular, antibodies and complement are ineffective. Once M. TB is phagocytosed, it can inhibit phagosome-lysosome fusion. The exact mechanism used by M. TB to accomplish this is not known but it is thought to be the result of a protein secreted by bacterium that modifies the phagosome membrane. Ca^{2}+ signaling is inhibited when *M. tuberculosis* enters human macrophages but not when killed *M. tuberculosis* or antibody-opsonized *M. tuberculosis* cells are phagocytosed (Malik *et al.,* 2000). This effect was correlated with trafficking to late endosomes; i. e., elevated Ca^{2}+ levels were associated with phagolysosome Ca^{2+} levels would help *M. tuberculosis* avoid

these host defense mechanisms. It has also been postulated that a selective advantage to *M. tuberculosis* of staying in an early endosome is that there would be less host immunosurveillance by $CD4^+$ T cells. In agreement with this idea, there is a decrease in the expression of major histocompatibility complex class II (MHC-I) proteins and in the MHC-II presentation of bacterial antigens in macrophages after *M. tuberculosis* infection (Noss *et al.,* 2001). This effect seems to be induced by presence of the secreted or surface-exposed *M. tuberculosis* 19-kDa lipoprotein, which is thought to interact with TLR-2 in the early phase of bacterial entry into macrophages (Thoma-Uszynski *et al.,* 2001). The mechanism by which virulent *Mycobacteria* prevent phagosomal maturation is not known, but in the normal maturation of the *Mycobacterial* phagosome there is a successive recruitment of Rab proteins, which are small GTPases involved in endosome trafficking; i. e., Rab5 associates with early endosomes, and Rab7 is found in later endosomes. The *M. tuberculosis* phagosome that does contain Rab5 does not recruit Rab7 (Via *et al.,* 1997). Also, TACO, a member of the coronin family of actin binding proteins, is preferentially recruited to the *Mycobacterial* phagosome of infected murine macrophages, where it was reported to be retained in phagosomes containing live and not killed *M. bovis* BCG (Ferrari *et al.,* 1999). The bacterium may remain in the phagosome or escape from the phagosome, in either case finding a protected environment for growth in the macrophage.

M. TB interferes with the toxic effects of reactive oxygen intermediates produced in the process of phagocytosis by two mechanisms:

1.Compounds including glycolipids, sulfatides and LAM down regulate the oxidative cytotoxic mechanism.

2.Macrophage uptake via complement receptors may bye-pass the activation of a respiratory burst.

Antigen 85 complex: This complex is composed of a group of proteins secreted by M. TB that is known to bind fibronectin. These proteins may aid in walling off the bacteria from the immune system and may facilitate tubercle formation although evidence of this is lacking.

Slow generation time. Because of M. TB's slow generation time, the immune system may not readily recognize the bacteria or may not be triggered sufficiently to eliminate them. Many other chronic disease are caused by bacteria with slow generation times, for example, slow-growing *M. leprae* causes leprosy, *Treponema pallidum* causes syphilis, and *Borrelia burgdorferi* causes Lyme disease.

High lipid concentration in cell wall, as mentioned previously, accounts for impermeability and resistance to antimicrobial agents, resistance to killing by acidic and alkaline compounds in both the intracellular and extracellular environment, and resistance to osmotic lysis via complement deposition and attack by lysozyme.

Cord factor: The cord factor is primarily associated with virulent strains of M. TB. It is known to be toxic to mammalian cells and to be an inhibitor of PMN migration. However, its exact role in M. TB virulence is unclear.

2.14 Anti-*Mycobacterial* agents

Drugs used to treat tuberculosis are classified as first-line and second-line agents. First-line essential tuberculous agents are the most effective and are a necessary component of any short courses therauptic regimens. The three drugs in this categories are a fat soluble complex, mycrocyclic antibiotic Rifampin (RIF), water soluble hydrazides of isonicotinis acid, Isoniazid (INH), and a derivative of nicotinic acid, Pyrazinamide (PZA). The first-line supplemental agents, which are a highly effective and infrequently toxic, include a water-soluble derivative of Ethylenediamine ethambutol (EMB) and an aminoglycoside isolated from S*treptomycine griseus*, Streptomycine (SM). Second-line anti-tuberculous drugs are much less effective than the first-line agents and much more frequently elicit severe reactions. They include a complex cyclic polypeptide derived from streoptomyces capriolus, Capriomycin, aminoglycosides like amikacin and kanamycin, para-amino-salisyclic acid (PAS), a type of isoniazid, thiacetozone a complex based polypeptide antibiotic, viomycin, a derivative of isonicotanic acid, ethionamide and amino derivative of isoxazolidinone, cycloserine. Newer anti-tuberculous drugs, which have not yet been placed in first-line essential anti-tuberculous agents, the first-line supplemental agents and second-line antituberculous drugs include a semi-synthetic Rifamycin supiropiperidly derivative, Rifabutin, a semisynthetic cyclopentyl rifamycin antibiotics, rifapentine and fluorinated quenolonse like Ofloxacin, Ciprofloxacin, Sparfloxacin and Pefloxacin. Because administration of a single drug often leads to the development of a bacterial population resistant to that drug, effective regimens for the treatment of TB must contain multiple drugs to which the organisms are susceptible.

When two or more drugs are used simultaneously, each helps prevent the emergence of tubercle bacilli resistant to the others. However, when *in vitro* susceptibility of a patient's isolate is not known, which is generally the case at the beginning of therapy, selecting two agents to which the patient's isolate is likely to be susceptible can be difficult, and improper selection of drugs may subsequently result in the development of additional drug-resistant organisms.

Hence, tuberculosis is usually treated with four different antimicrobial agents. The course of drug therapy usually lasts from 6-9 months. The most commonly used drugs are rifampin (RIF) isoniazid (INH), pyrazinamide (PZA) and ethambutol (EMB) or streptomycin (SM). When adherence with the regimen is assured, this four-drug regimen is highly effective. Based on the prevalence and characteristics of drug-resistant organisms, at least 95% of patients will receive an adequate regimen (at least two drugs to which their organisms are susceptible) if this four-drug regimen is used at the beginning of therapy. Furthermore, a patient who is treated with the four-drug regimen, but who defaults therapy, is more likely to be cured and not relapse when compared with a patient treated for the same length of time with a three-drug regimen.

2.15 Cell Wall

The shape forming properties of the bacterial cell walls are attributing to the peptidoglycan, whose chemical structure in *M. tuberculosis* closely resembles that found in other bacteria. Attached to this by phosphodiester bond is a branched chain polysaccharide, the arabinogalctan, whose distal ends are

esterified with high-molecular weight fatty acid (mycolic acids) of sizes and structure unique to *Mycobacteria.* Mycolic acids are 1-alkyl branched 2-hydroxy fatty acids, typically with 70 to 90 carbon atoms. The branch is commonly about 24-carbon atom long and is a simple alkyl chain, but the main chain contains (*M. tuberculosis*) cyclopropyl, Methoxyl or keto and methyl groups. This asymmetry between the two-alkyl chains is important in the construction of models of *Mycobacteria*l envelope. Peptidoglycan-arabinogalctan Mycolate forms the so-called cell wall skeleton, which is readily isolated and studies associated with the skeleton, which is a large variety of lipids and glycolipids (and possibly some proteins). These difference considerable among *Mycobacterial* species or group of species and a few have highly distinctive ultra structural appearances, but those associated with *M. tuberculosis* (many of which are well studied and well understood from a chemical point of view) are not among the ultra structurally recognizable types. None of these associated molecules is the anatomical situation in *M. tuberculosis* known and their connections with ultra structure are not definitely known. New wall-associated molecules continue to be described and it seems certain that some remain to be isolated and identified. Consequently, one must admit the possibility that some features of the appearance of the wall are caused by substances still unknown.

The special ability of *M. tuberculosis* to survive in a mammalian host and to cause a potentially fatal disease presumably derives at least in part from the nature and arrangement of particular chemicals in the bacterial cell. Further

study of the ultrastructure of this particular species is needed to understand its pathogenic properties.

2.15.1: Cell envelope

The *Mycobacteria* cell envelope contains high proportion lipids, whose diverse structures have obvious chemo systematic potential. It is lipid part of cell wall that gives *Mycobacteria* their unusually thick and waxy envelop. The elaborate and distinctive feature of many of the cell wall moieties has led to speculate that these are involved in the virulence and pathogenesis of *Mycobacterium*. These structure include mAGP, LAM, LM, PIM, SL trehalose, 6,6'-dimycolate (cord factors), other acylated trehalose, phenolic glycolipid, lipooligosaccharide and attenuation indicator lipid. While some of these molecules, such as SL and cord factor, have previously been implicated in host-pathogen interaction (Goren and Brennan, 1979).

Peptidoglycan forms the basal layer of cell wall to which arabinogalctan and in turn, Mycolate are covalently attached. The peptidoglycan in *Mycobacteria* is of a common in many bacteria (Draper, 1982; Dean *et al.,* 2001) but with two slight differences. First, there are interpeptide linkages between two diaminopimelate residues as well as the usual D-alanyl-diaminopimelte linkages (Wietzerbin *et al.,* 1975). Second, the usual N-acetyl-muramic acid is replaced with N-glycolyl-muramic acid *M. bovis* and in other *Mycobacteria* (Azuma *et al.,* 1970).

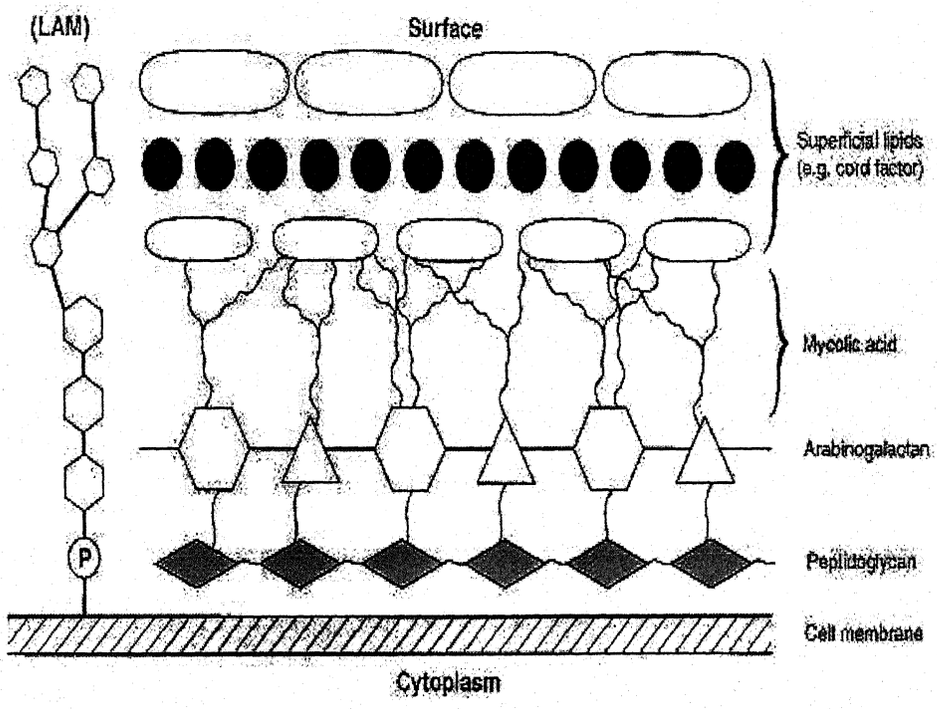


Figure 6: Complex cell wall structure of *Mycobacteria.*

2.16: Clinical identification and prevention

Diagnosis of tuberculosis is a high index of suspicion. Diagnosis is not difficult with a high-risk patient. On the other hand it can easily be missed in a patient with a focal infiltrate. Diagnosis of tuberculosis requires detection of acid-fast bacilli in appropriate specimens depending upon the type of tuberculosis. Sputum is the most common specimen submitted for culture when pulmonary tuberculosis is suspected. Although not as satisfactory as expectorated sputum, induced sputum is a better specimen than gastric aspirates for the recovery of *Mycobacteria.* Bronchoscopy specimens or transtracheal aspirations are also appropriate respiratory tract specimens for *Mycobacterial* culture. In case of extra pulmonary tuberculosis appropriate specimens include blood, pleural fluid, bronchial, aspirates, bone marrow, purulent exudates, joint fluid, cerebrospinal fluid, nasopharyngeal swab and others. The examination of feces is usually not of value, since cases of intestinal tuberculosis are extremely rare. However, in patients with the acquired immune deficiency syndrome (AIDS) acid-fast stain and cultures of feces may be necessary for the efficient detection of *Mycobacteria* (Hart and Sutherland, 1977). M. TB can be cultured from specimens for definitive diagnosis. *Mycobacteria* have an extended generation time (20 - 22 hours) when compared with those of common bacterial species (40 – 60) which may appear in respiratory tract specimens. Therefore, *Mycobacteria* may be rapidly over grown by other contaminating bacteria if the latter are not inhibited. Also, bacteria waste products may accumulate in the medium and potentially inhibit the growth of *Mycobacteria.*

The high lipid content of *Mycobacterial* cell walls provides more resistance to strong acids and alkalis than is found with other bacteria. This resistance has promoted the development of decontamination procedures used to eliminate most common bacterial flora while maintaining the viability of *Mycobacteria.*

A number of alkaline digestion, decontamination solutions are commonly used for eliminating bacteria from contaminated specimens. Sample treatment with NaOH kills other contaminating bacteria but does not kill the M. TB present because M. TB is resistant to alkaline compounds by virtue of its lipids layer.

2.16.1: Growth Requirement

Myobacteria are among the least fastidious of the pathogenic microorganisms. Growth requirements on artificial media include potassium, magnesium, phosphorus and sulfur. Nitrogen requirements are supplied by ammonium salts or whole egg ingredients and carbon requirements are supplied by glucose or glycerol. *Mycobacteria* grow best in a pH range of 6.5 to 7.0.The bacteria is strict aerobe and require proper aeration for the elaboration of certain enzymes and pigments. A CO_2 concentration from 5% to 10% is necessary for the primary recovery of *Mycobacterium tuberculosis* and other *Mycobacteria.* Carbon dioxide is slowly produced during growth of the culture; however, a CO_2 atmosphere is required for the first 3 to 4 weeks of incubation. Optimum culture conditions for *M. tuberculosis* also include a relatively high humidity and an incubation temperature of 35° to 37° C.

Four different types of traditional culture media used for the recovery of *Mycobacteria* are shown below and include egg-based, agar-based, liquid and

selective media. Egg-based media include the following ingredients: whole eggs, potato flour, salts, glycerol and varying concentration of malachite green, an inhibitory dye. This medium type does not contain agar but is solidified by heating to 85° to 90°C for 30 to 45 minutes (inspissations).

The selection of media for the recovery of *Mycobacteria* is important. Most laboratories chose a combination of traditional egg-based, agar-based and /or selective media for the optimal recovery of *Mycobacteria.* Although many combinations exist, an optimal one would include L-J, 7H9 and S7H11 medium, the BACTEC-460, Middlebrook 7H12 and Souton's medium. Because most species of *Mycobacteria,* including *M. tuberculosis*, grow slowly, 4 to 8 weeks may be required before growth is detected. Although *M. tuberculosis* may be presumptively identified on the basis of growth time and colony pigmentation and morphology, a variety of biochemical tests have traditionally been used for identification of *Mycobacteria*l isolates. In today's laboratories, the use of liquid media with radiometric growth detection (e. g. BACTEC-460) and the identification of isolates by nucleic acid probes or high-pressure liquid chromatography of mycolic acids have replaced the traditional methods of isolation on solid media and identification by biochemical tests. These new methods have decreased the time required for isolation and speciation upto 2 to 3 weeks other systems for culture on liquid media non-radiometric detection have become available.

The commonly used automated technique, the BACTEC system developed by Johnston Laboratories Cockeysville, MD for rapid detection of *Mycobacterial*

growth in clinical specimens used liquid Middle Brook 7H12 medium containing ^{14}C labeled palmetic acid for the radiometric detection of *Mycobacterial* growth. The ^{14}C label is evolved as $^{14}CO_2$ during growth and respiration. When the *Mycobacterial* growth reaches a predetermined growth index of 10, as determined by the amount of $^{14}CO_2$ evolved; a positive result is recorded by the BACTEC system. M.TB growth can be detected in 9-16 days vs 4 - 6 weeks using conventional media. The presumptive diagnosis is commonly based on diagnosis of *Mycobacteria* based on finding of AFB (Acid-fast bacillus) on microscopic examination of diagnostic specimens. The presence of AFB does not provide information concerning the identification or viability of the organism, since all species of *Mycobacteria* are acid-fast. (Textbook of Bacteriology. 2005; Minnikin *et al.,* 1984). Most modern laboratories are processing large numbers of diagnostic specimens using two acid-fast control-fuchsin methods, including the Ziehl-Neelson (Z-N) and Kinyoun procedures and a fluorochrome method using auramine o or auramine- rhodamin dyes.

2.16.2: Diagnosis

Purified Protein Derivative (PPD) skin testing: PPD is most widely used as screening for *M. tuberculosis* infection and it is performed as the tuberculin or Mantoux test. Skin Testing is performed as the tuberculin or Mantoux test. PPD is employed as the test antigen in the Mantoux test. PPD is generated by boiling a culture of M. TB, specifically old Tuberculin (OT). 5 TU (tuberculin units), which equals 0.0001 mg of PPD in a 0.1 ml volume is subcutaneously injected in the forearm. The test is read within 48-72 hours. The test is

considered positive if the diameter of the resulting lesion is 10 mm or greater. The lesion is characterized by erythema (redness) and swelling and induration (raised and hard). 90% of people that have a lesion of 10 mm or greater are currently infected with M. TB or have been previously exposed to M. TB 100% of people that have a lesion of 15 mm or greater are currently infected with M. TB. or have been previously exposed to M. TB (Kenneth Todar, Bacteriology. 2005). The test is of limited value in the diagnosis of active tuberculosis because of its low sensitive and specificity.

Sensitization with nontuberculous bacteria leads to false-positive results and the test is difficult to interpret. A positive tuberculin test may be due to active tuberculosis, past infection, BCG vaccination or sensitization by environmental *Mycobacteria*. Therefore, this test is more helpful in places where BCG vaccination is no longer used effectively (Garg *et al.* 2003).

False negatives are more rare than false positives but are especially common in AIDS patients as they have an impaired CMI response. Other conditions such as malnutrition, steroids, etc., can rarely result in a false negative reaction. Recently, a recombinant antigen (DPPD) encoded by a gene unique to the *M. tuberculosis* complex organisms proves to be better than PPD in the Mantoux test (Campos-Neto *et al.*, 2001). It can facilitate a more specific diagnosis of tuberculosis since the DPPD gene is not present in nontuberculous bacilli.

A number of nontraditional diagnostic tests have been evaluated as adjuncts to above mentioned traditional standard laboratory diagnosis. The

most thoroughly investigated is based on a number of *Mycobacterial* proteinaceous (ESAT-6, 14kDa, MPT63, 19kDa, MPT64, and 38kDa) and non-protienaceous antigens (Trehalose based glycolipids like cord factor, trehalose-6, 6' dimycolate, acytrehaloses and phenolglycolipids) and detection of antibody *Mycobacterial* antigens in specimanse of involved sits (e. g. sputum for pulmonary, CSF for tuberculous meningitis, pleural fluid and biopsy samples for pleural disease) and also in bone marrow and liver biopsy,(Daleine, 1995; Garg *et al.,* 2003). This serological diagnosis has generally not been sufficiently sensitive to be clinically useful. It has been difficult to develop an ELISA utilizing a suitable antigen because *M. tuberculosis* shares a large number of antigenic proteins with other microorganisms that may not be pathogenic. Nonspecific tests, such as the measurement of adenine deaminase in pleural fluid, have been evaluated but have not gained acceptance.

Recently, the γ-interferon assay was assessed as a potential candidate to replace the Mantoux skin test. The assay was evaluated in groups of immigrants, health-care workers and *M. tuberculosis* and *M. avium* complex MAC patients. The efficacy of the Mantoux test in cases effective tuberculosis, and it detected three of the seven cases of MAC colonization (Bellete *et al.,* 2002). New generation of diagnostic test that are based on molecular biology techniques are helping to rectify existing flaws in tuberculosis diagnosis but tend to be too costly for use in low-income setting. The approaches based on molecular biology for tuberculosis diagnosis includes branched DNA, Signal amplification (Vareldziz, 1994), Strand displacement amplification (SDA)

(Wolinsky and Schaefer, 1973; Walker *et al.,* 1992), Polymarese chain reaction (PCR) (Pottumarthy *et al.,* 2000; Mileler *et al.,* 1994; Altamirano *et al.,* 1992; Eisenach *et al.,* 1990; Verbon *et al.,* 1990; Hance *et al.,* 1989), Ligas chain reaction (LCR) (Jacob *et al.,* 1993; De Wet *et al.,* 1987), DNA amplification, Transcription mediated amplification (TMA) *(Jonas et al.,* 1993; Gladwin *et al.,* 1998), and utilization of constructed Reporter *Mycobacterium* phage (Jacob *et al.,* 1993; De Wet *et al,*1987).

2.16.3: Prevention

BCG vaccine

In 1908, Camille Guérin and Albert Calmette initiated their attempts to produce an anti-TB vaccine from a virulent bovine strain. In 1921, vaccination with BCG, an attenuated vaccine, was introduced (Sakula, 1983). The efficiency of the BCG vaccine has been questioned since its early use and therefore, a large number of trials have been carried out to determine its efficacy. In these studies it was found that, the BCG vaccine protected efficiently against leprosy (Fine and Rodrigues, 1990) as well as childhood manifestations of TB (disseminated TB) (Rodrigues *et al.,* 1993). However, the protective efficacy against pulmonary TB was limited (Tuberculosis Research Centre (ICMR), Chennai, 1999).

Many hypotheses have been suggested to explain the low protective efficacy of BCG against pulmonary TB. These hypotheses include inappropriate treatment and storage of the vaccine, the use of different strains of BCG (Fine, 1995), and lack of an effective stimulation of the optimal blend of T cell populations and in particular that of the $CD8^+$ T cells (Hess and Kaufmann,

1999). In addition to these hypotheses, the currently used intradermal route of immunization has been suggested as another factor influencing the capacity of BCG to induce optimal immunity in the lungs. In this regard, (i. n.) route of immunization has recently been evaluated as a possible route for BCG delivery, in mouse experimental models. Results from this study showed a high degree of protection against challenge with *M. tuberculosis* in BALB/c mice, following BCG vaccination (Falero-Diaz *et al.,* 2000). In a similar model, vaccination with BCG conferred as good, if not better protection than subcutaneous (s. c.) route, against challenge with virulent *M. bovis* (Lyadova *et al.,* 2001).

2.17.3: Prospects for new vaccines

Given the limitations of BCG in protection against adult pulmonary TB, there is a considerable scope for improved vaccination strategies. Immunological research has a key position in understanding the pathogenesis of TB, and thereby in developing novel designs for effective prophylactic vaccination, immunodiagnostic tools and immunotherapeutic agents. Two approaches have been considered for vaccine development. One involves the replacement of BCG by a more potent vaccination inducing immune responses capable of either complete elimination of the bacilli, or of reliable containment of persistent infection.

The second approach involves the post-exposure vaccination to boost immunity in individuals whose natural immunity has already been primed by infection or BCG vaccination (Young and Stewart, 2002). Indeed, over the past decade research efforts have been directed to evaluate potential vaccine

candidates as well as alternative routes of vaccine delivery, such as the i. n. route, in order to improve protection.

2.16.4: New vaccine candidates

A wide range of potential vaccine candidates have been generated and subjected to tests for protective efficacy in experimental model of infection. New vaccine candidates include live attenuated vaccines, subunit vaccines and DNA vaccines.

2.16.5: Live attenuated vaccines

Advances in the techniques required to genetically modify *Mycobacteria*, as well as the increase in the knowledge of the pathogenesis of the microorganism, have made possible to delete genes encoding for potential virulence factors in *M. tuberculosis*, thereby enabling the generation of attenuated mutants. In addition to attenuated strains of *M. tuberculosis*, natural attenuated *Mycobacteria*, such as *M. vaccae* and *M. microti*, are being studied as possible vaccine candidates (Nor and Musa, 2004). Another approach has been the improvement of the BCG immunogenicity by the addition of genes encoding cytokines, such as IFN-γ (Murray *et al.*, 1996) or *Mycobacterial* proteins, such as the antigen 85 complex (Ag85) (Horwitz *et al.*, 2000).

Although encouraging results have been obtained in challenge experiments (Horwitz *et al.*, 2000; Smith *et al.*, 2001), a major consideration for the clinical use of live vaccines is safety, specificity when considering TB vaccination strategies for AIDS patients.

2.16.5.1: Subunit vaccines

Subunit vaccines are currently the most widely studied. This type of vaccine has been focused in particular on proteins present in filtrates prepared from *in vitro* cultures of *M. tuberculosis*, although non-secreted antigens have also been shown to induce protective responses in experimental studies (Coler *et al.,* 2001; Skeiky *et al.,* 2000).

The most extensively studied antigens are members of the Ag85 complex, a family of mycolyl transferases enzymes involved in cell wall biosynthesis and present in culture filtrates (Belisle *et al.,* 1997). The Ag85 has been reported to induce strong activation of T cells in several studies (Andersen *et al.,* 1995; Mustafa *et al.,* 1998). Other antigens being studied are:

(i) Early secreted antigenic target (ESAT-6), which has been reported to be absent from all BCG vaccine strains and to induce very strong T cell and antibody responses (Brodin *et al.,* 2004).

(ii) Heat-shock proteins (HSP), such as HSP-65 and HSP-70, found to induce a prominent immune response at both, the antibody and the T cell levels (Silva, 1999).

(iii) PstS-1 (38 kDa protein), a glycoprotein exposed on the surface of the bacillus and reported to be a powerful B and T cell antigen (Bothamley *et al.,* 1992; Lefevre *et al.,* 1997).

(iv) 19 kDa protein, a lipoprotein found to induce the expression of IL-12 and iNOS inmonocytes and dendritic cells through its binding to TLR2

(Brightbill *et al.,* 1999;Thoma- Uszynski *et al.,* 2000) and to promote neutrophil activation (Neufert *et al.,* 2001).

A limiting factor of the subunit vaccines is the need of adjuvant for vaccine delivery. Currently research studies are focused on the choice of which adjuvant to use and whether immunomodulatory, such as cytokines, should be used. Despite this drawback, subunit vaccines based on recombinant protein antigens are attractive because the techniques for production are established and this type of vaccine is expected to satisfy the regulatory requirements for use in humans more easily than the live vaccines.

2.16.5.2: DNA vaccines

Administration of naked DNA has the potential of eliciting both, cellular and humoral immunity against encoded antigens. Several *Mycobacterial* antigens, including the PstS-1, HSP-65 and the Ag85 have been studied and found to induce protection in animal models (Bonato *et al.,* 1998; Fonseca *et al.,* 2001; Huygen *et al.,* 1996). Although the results are promising, concerns about the safety of DNA vaccination have been raised, mainly regarding the possibility of DNA integration into the host genome affecting oncogenes or tumor suppressor genes and thereby inducing the development of cancer. However, the risk of integration has been reported to be low under a variety of experimental conditions (Manam *et al.,* 2000; Martin *et al.,* 1999).

2.16.5: Experimental animal models in TB

Discussions about the value of experimental animal models in TB research have a longstanding history. Experimental animal models are critical for

delineating the general mechanisms underlying natural resistance, and acquisition of a protective immune response against TB. However, assessment of this information using experimental animals should be conducted carefully since there are differences in the host defense mechanisms between experimental animals and humans.

Many experimental animal species such as mouse, guinea pig, and non-human primates, have been used for deciphering the mechanisms involved in TB. The mouse, without doubt, is a very sophisticated and cost-efficient model. The immune response of the mouse is very well understood, and reagents such as monoclonal antibodies against surface antigens and cytokines are available. More importantly, the genetic manipulation of this species is highly advanced. Trans gene expression, gene knockout, gene knock-in have all become standard technologies, and a large variety of mouse mutants with defined immunodeficiencies are available to researchers studying the role of distinct cells and effector molecules in the *in vivo* setting of TB. Moreover, the recent elucidation of the murine genome promises to open a new area of research with enormous impact on our understanding of genetic disorders and also of host mechanisms in TB (Kaufmann, 2003).

It is now well evident that tuberculosis poses a significant health rate threat to mankind. Multidrug resistant strains are on the rise, and *Mycobacterium tuberculosis* infection is often associated with human immunodeficiency virus infection. Satisfactory control of tuberculosis can only be achieved using a highly efficacious vaccine.

Currently, two main vaccination strategies are being pursued. The first strategy uses subunit vaccines in the form of protein-adjuvant formulation, naked DNA, or recombinant bacterial or viral carriers that express defined antigens. Promising results have been obtained, but so far no vaccine candidate tested in animal models has proven to be better than BCG. The second strategy, comprising viable *Mycobacterial* vaccines, either attenuated viable *M. tuberculosis* or BCG, or recombinant BCG over expressing certain antigens or immunomodulatory, is also being pursued and shows promise.

Recently the attentions have been focused on secretoty protein antigens of *Mycobacterium*, which are synthesized by the actively growing *M. tuberculosis* culture and to induce the desired immune response (Anderson and Heron, 1993; Anderson, 1994). These proteins have also been termed as culture proteins and known to elicit strong immune reaction in humans and animals infected with *M. tuberculosis*/ *M. bovis* (Anderson *et al.,* 1991 b; Orme *et al,* 1992; Romain *et al.,* 1993; Anderson, 1994).

As a result of the combined efforts of several laboratories, more than 30 secretory proteins of *M. tuberculosis* have been characterized, (Andersen *et al.,* 1991; Anderson, 1994, Kamath *et al.,* 1999; Sonnenberg and Belisle, 1997; Karin *et al.,* 1998; Gennaro, 2000; Kanaujia *et al.,* 2004; Spencer *et al.,* 2004; Young *et al.,* 2004; Sable *et al.,* 2005). These proteins are known to elicit strong immune reactions in humans infected with *M. tuberculosis / M. bovis* (Anderson *et al.,* 1991; Orme *et al.,* 1992; Romain *et al.,* 1993; Anderson 1994; Ingrid *et al.,* 2000; Sable *et al.,* 2005).

The secretory proteins have been demonstrated to be strongly recognized by T-cells isolated from human (Tuberculosis) TB patients (Orme, 1996; Spencer *et al.,* 2004) as well as mice and cattle experimentally infected with tuberculosis (Anderson and Heron, 1993; Pollock and Anderson, 1997; Lanbo *et al.,* 2004). Experimental work in animal models suggests that both CD_4^+ and CD_4^+ T cells are required for optimal protection against tubercle bacillus (Orme *et al.,* 1992; Bonato *et al.,* 1998; Flynn *et al.,* 1992; Pais *et al., 1998;* Spencer *et al.,* 2004).

These proteins have been the focus of much of the research directed at identifying antigens that induce protective immunity or those that elicit immune responses of diagnostic value (Aub *et al.,* 2000; Lein *et al.,* 1999; Young *et al.,* 2004; Paolo *et al.,* 2004).

The recent identification of novel secreted proteins of *M. tuberculosis* open the way to studies on their immunological characterization of these protein to define their potential for immonological diagnosis of tuberculosis or vaccine design.

A few numbers of secretory antigenic protein and peptides from M. TB have already been evaluated as antigens for the immunodiagnosis of tuberculosis. In an effort to develop more accurate diagnostic tools, and suitable vaccine the present research program is aimed to assess the diagnostic and preventive potential of certain secretory candidate proteins.

The *M. tuberculosis* culture filtrate, which contains as many as 200 proteins (Romain *et al.,* 1993; Ingrid *et al.,* 2000), has been investigated by means of protein purification by immunological methods, and by screening of expression libraries of *M. tuberculosis* DNA with anti-culture filtrate sera (Wolinsky and Schaeferm, 1973; Ginsberg, 1998; Grange and Laszlo, 1990; Bothemley *et al.,* 1991; Altamirano *et al.,* 1992; Sorensen *et al.,* 1995; Mileler *et at.,* 1994; Bellete *et al.,* 2002).

The early secretory antigenic target (ESAT)-6, purified protein and peptides from *Mycobacterium tuberculosis* have been already under evaluation as antigens for the immunodiagnosis of tuberculosis (TB). Some important antigens, the (38 KDa, 30/31 KDa, 40 KDa, 42 KDa, SOD, 30 KDa MSP, 85B, ESAT-6, and CFP10) molecules, have been found to be secreted by *Mycobacterium tuberculosis* (Roberts *et al.,* 1995: Ulrichs *et al.,* 1998; Gennaro, 2000: Smith, 2003: Spencer *et al.,* 2004). These *Mycobacterial* antigens are already under process in various laboratories to use as markers for tuberculosis diagnosis by using ELISA and other non-traditional diagnostic technology.

As we are aware of effect that one of the main objectives of the research in the field of *Mycobacteriology* is the development of new methods that will improve and expedite the diagnosis and treatment of tuberculosis and other *Mycobacterial* infections. Some forms of tuberculosis are difficult to diagnose by the available routine diagnostic methods. In spite of new technologies, no reliable new serological test has been developed for the diagnosis of

tuberculosis. No reports are available on the diagnostic and protective efficacy of low molecular weight M. TB secretory proteins for patients suffering with TB. Therefore present research has been investigated for identification of low molecular weight *Mycobacterial* antigenic secretory proteins and development of immunodiagnostic tool for detection and diagnosis of tuberculosis.

The study has been focused towards the following properties with special attention- High sensitivity, High specificity, Reproducibility and Stability.

This will be simplify the effort towards the development of more efficient immunodiagnostic system, effective vaccine and immunotherapy for the prevention and therapy of tuberculosis although diagnostic system based on diverse native epitopes are developed but a continuous effort is required to further evolve more and more efficient diagnostic tools.

Research design to apply isolated and screened low molecular weight secretory proteins with special reference to immunoprophylactic activity and development of a novel diagnostic tool, by using searched antigenic low molecular weight secretory protein to detect anti-*Mycobacterial* antibodies by various possible designed tools in the form of Tri Dot/ ELISA/ Bi Spot/ Immuno-chromatography, finally the developed diagnostic product will be evaluated to find out its applicability and acceptability under following: Sensitivity, Specificity, and Feasibility in the field and immuno protective for subunit vaccine.

3

Aims and Objectives

AIMS

The present study was undertaken to identify and purify the immunoreactive low mass of secretory proteins of *M. tuberculosis* and to study their immuno protective potential against experimental tuberculosis for the development of safe and effective vaccine and also to explore the potential of some of these protein antigens for the development of effective diagnostic tool.

OBJECTIVES

1) To optimize the conditions of harvesting of culture filtrate proteins for the isolation of secretory proteins of *M. tuberculosis*.
2) To purify major secretory proteins of *M. tuberculosis* using chromatographic and electrophoresis techniques.
3) To optimize and standardize the conditions for perfoming ELISA to titrate the antibody / antigens of *M. tuberculosis*.
4) To study the cellular and humoral response elicited by the purified antigens of *M. tuberculosis*.
5) To study the protective efficacy of most immunoreactive secretory proteins of *Mycobacterium tuberculosis* against *M. tuberculosis* infection in mouse model.
6) To obtain a cocktail of secretory proteins as antigens relevant for serodiagnosis, since no single antigen is known to represent whole tuberculosis population.
7) To develop simple, and more reliable methods for diagnosis of pulmonary as well as extra-pulmonary tuberculosis.

4

Material and Methods

4.1.1: Chemicals used

All chemicals, media, dye and stains used throughout the study were obtained from standard companies and were of AR / LR grade high quality pure chemicals. Briefly some of the chemical used were Acrylamide (Sigma), Arginine (s. d. fine), Acetic acid (s. d. fine), Ammonium persulphate (Sigma), Ammonia (Sigma), Bromophenol blue (Sigma), Bis-acrylamide (Sigma), N,N,N',N'-Tetramethylethylene diamine (Sigma), SDS (Sigma), Mercaptoethanol (Sigma), Brilliant blue–R (Sigma), Methanol–LR (s. d. fine), Silver Nitrate (Qualigens fine), Formaldehyde (Qualigens fine), Citric acid (Qualigens fine), Sodium chloride (Qualigens fine), Glycine (Qualigens fine), Bovine Serum Albumin (Genei),3,3', Diaminobenzidine (DAB) (Sigma), Nickel chloride (Qualigens fine), di- sodium phosphate (Merck), Sodium dihydrogen phosphate (Merck), Sodium acetate (s. d. fine), Glycerol (s. d. fine), Nitrocellulose transfer membrane (Pall Scientific, USA), Goat Anti- human IgG-HRP conjugate (Genei), Tween 20 (Sigma), Cupric sulphate (Qualigens fine), Casien digest (CD), Amicon membrane (Pall Scientific, USA), DEAE-Sepharose (Pharmacia), Protein molecular weight standards Marker (Amershan pharmacia biotech), HRPO-Anti-rabbit IgG conjugate (Dako, USA), Glucose (Qualigens fine), Colour substrate solution (Bangalore Genei), 7H9 medium (Difco USA), Sauton's Medium (Qualigens fine), Growth supplement pyruvate (Qualigens fine), Asparagine (Himedia), Tricine (Merck).

4.1.2: Instruments / Equipments used

Shaker, Vacuum pump, Fume hood, Freezer, Ultracentrifuge, Table top centrifuge, Electrophoresis unit, Western blot unit, Spectrophotometer, ELISA

reader, Chromatography Columns, Ultra filtration assembly, BOD Incubator and CO_2 Incubator etc.

4.1.3: Bacterial Strain

Mycobacterium tuberculosis $H_{37}Rv$ (ATCC-27294) was obtained in frozen form ($2x10^9$ cfu/ml) from Dr V. M. Katoch, Director, Central JALMA Research Institute for Leprosy, Agra, India, on an LJ slant. This strain was further grown in one Litre of Sautons' media at 37°C for 50 days till late log phase. The growth was collected by centrifugation at 4°C, 10,000g for 20 minutes and pellet was suspended in 100 ml of PBS (pH 7.2) to wash the bacterial pellet. Harvested cells were resuspended in physiological saline and adjusted to opacity of 1.0 Mac Farland and transferred to sterile eppendroff tubes in 1.0 ml quantity and stored at –70°C till further use.

4.2: Culture of Bacteria and Measurement of Protein Secretion

All the procedures were conducted within a Biological Safety Cabinet class II, and class III Laboratory.

4.2.1: Media Preparation

Media used for maintenance (LJ medium slants) and growth (sautons medium) of *M. tuberculosis* $H_{37}Rv$ was prepared as under: -

(a) Lowenstein Jensen (LJ) medium (Jensen1932)

(i) Mineral salt solution

Ingredient	Quantity
Potassium dihydrogen phosphate (anhydrous)	2.4g
Magnesium Sulphate	0.24g
Asparagine	3.6g
Glycerol	12.0 ml
DH_2O	600.0ml

The above ingredients were dissolved by heating and autoclaved at 121°C for 15 minutes.

(ii) Malachite green solution

A two percent solution was prepared in sterile distilled water by dissolving the dye at (37°C) for 1-2 hrs.

(iii) Preparation of complete media

Ingredient	Quantity
Mineral salt solution	600.0 ml
Malachite green solution	20.0 ml
Egg yolk	1000.0 ml

For preparation of media all ingredients were sterilized. Eggs were taken fresh (not more than 7 days old) and washed with clean distilled water and dried with whatman No. 1 filter paper. Eggs were cracked with a sterile scalpel in a conical flask having glass beads. Beaten eggs were filtered through 3-mm cotton gauge. The complete medium was mixed and distributed in 10-ml screw capped bottle and screwed tightly. Bottles were laid horizontally in an inspissator and heated at 80°C for 1 hr. Process of heating was repeated for two more subsequent days.

(b) Sautons Liquid medium (Sautons-1912)

Ingredients	Quantity
Ferric ammonium citrate	0.05g
Potassium dihydrogen phosphate	0.5g
Magnesium sulphate	0.5g
Citric acid	2.0g
Asparagine	4.0g

Dissolved all salts in 200 ml of distilled water by heating at 70°C, pH was adjusted to 7.2 using 6.7 ml 5M NaOH, after addition of 60 ml of glycerol, volume was adjusted to one litre. The medium was autoclaved at 121°C for 15 minutes.

The entire flasks and other glassware were sterilized in a hot air oven at 160°C for 2 hours before being used.

4.2.2: Cultivation for harvesting of protein

Mycobacterium tuberculosis $H_{37}Rv$ was inoculated by loop (roughly 10 mg moist weight of bacteria) in to 250 ml of Sautons medium (Sauton, 1912) in 500ml flask. The flask was kept at 37°C in stationary condition and grown for 5-6 weeks (mid log phase). The bacterial growth was monitored spectrophotometerically by measuring at O. D. A_{580} nm against Phosphate buffer (10mM, pH-7.2).

4.2.3: Protein yield during growth

The culture (5.0ml) was collected after every 48 hours till 6 weeks for estimation of total protein content in the supernatant, For this the culture was centrifuged at 12,000 rpm for 30 minutes at 4°C supernatant was collected & sterile filtered. Supernatant was inactivated by formaldehyde (0.1µg/ml. The protein content was determined at A_{280} by spectrophotometer.

4.2.4: Analysis of protein profile by SDS-PAGE

The protein profiles of total culture filtrate and in different fraction obtained from column chromatography were analyzed by SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) as described by Lammeli (1970).

4.2.4.1 Reagents

- *Polymerizing (Acrylamide solution (30%))*

  The solution was prepared by dissolving 29.2 gms of acrylamide (Sigma) and 0.8gm of N, N-methylene-bis acrylamide in 100ml double distilled water in brown bottles and stored at room temperature till use.

- *Resolving / separating gel buffer*

  Solution was prepared by dissolving 27.23 gm of Tris buffer in 80 ml of double distilled water; the final pH was adjusted to 8.8 with 1N HCl and volume was made to 150ml with double distilled water. It was stored at 4°C till use.

- *Stacking gel buffer*

  This solution was prepared by dissolving 6.0 gm of Tris buffer in 60 ml double distilled water and adjusted the pH to 6.8 and made the final volume to 100 ml with double distilled water and stored at 4 ^{O}C till use.

- *Sodium Dodecyl sulphate solution*

  The solution was prepared by dissolving 1.0 gm of sodium dodecyl sulphate in 100 ml double distilled water and was stored at room temperature.

- *Ammonium persulphate (10%w/v) solution*

  Dissolved 10 mg of ammonium persulphate in 1.0ml distilled water and stored at 4°C.

  Sample buffer:

  Sample buffer was prepared by mixing Tris HCl (1.0ml), Glycerol (8.0ml), 10% SDS (1.6ml) and β-mercaptoethanol (0.4ml). The solution was stored at 4 ^{O}C.

- *Tris Glycine (running buffer)*

  The solution was prepared by mixing 0.1% SDS solution in 0.025 M HCl buffer (pH8.3) containing 0.192 M Glycine.

- *Staining solutio:*

  0.2% solution of Coomassie blue was prepared in solution containing methanol, glacial acetic acid and water in a ratio of 5:1:4.

- *Destaining and fixing solution*

  The solution was prepared by mixing methanol: acetic acid: distilled water in a ration 2:1:7.

4.2.4.2: Recipe for separating gel

The separating gel solution of different strength for mini gel was prepared as shown in table below using above-mentioned reagent.

Separating gel of different strength

Stock	5%	6%	7%	7.5%	8%	9%	10%	12%	13%	15%
Polymerizing solution	2.5	3.0	3.5	3.7	4.0	4.5	5.0	6.0	6.5	7.5
Separating gel buffer	3.65	3.65	3.65	3.65	3.65	3.65	3.65	3.65	3.65	3.65
Distilled H_2O	8.7	8.25	7.75	7.5	7.25	6.75	6.25	5.25	4.75	3.75
SDS (10%)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
APS (10%)	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
TEMED	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01

4.2.4.3: Recipe for stacking gel

Stock	Volume (ml)
Tris buffer (pH6.8)	1.25
Polymerizing solution	1.0
Double distilled water	2.7
SDS (10%)	0.1
APS (10%)	0.05
TEMED	0.005

4.2.5: Procedure

The mini electrophoresis unit (BIO RAD laboratories) was used to cast and run SDS-PAGE. Briefly, the gel plates along with the spacers were fastened onto the gel-casting stand. Solutions for resolving / separating gel were mixed carefully and poured between the two plates, leaving sufficient space for the stacking gel (the length of the teeth of the comb plus 1 cm). The 12% acrylamide: bis-acrylamide mix was overlaid with water to prevent oxygen diffusion into the gel which otherwise inhibits polymerization. After the polymerization is complete, the overlay was poured off and top of the gel was washed with deionized water to remove any unpolymerized acrylamide. Stacking gel solution containing 5% acrylamide: bis-acrylamide mix was mixed and poured directly onto the surface of the polymerized resolving / separating gel. A thin Teflon comb was immediately inserted into the stacking gel solution being careful enough to avoid air bubbles. After the polymerization is complete, comb was removed and wells were washed with deionized water to remove unpolymerized acrylamide. The gel was mounted in the electrophoresis apparatus and Tris-glycine electrophoresis buffer was added to the top and bottom reservoirs. Protein samples were prepared by adding 2XSDS sample buffer in 1:1 ratio. The mixture was boiled for 5 minutes at 100°C in and each well 40µl of sample containing 10-70 µg protein was loaded. Electrophoresis apparatus was attached to an electric power supply. The gel was run at constant current of 30mA for stacking gel and at 40mA in separating gel until the bromophenol blue reaches the bottom of the resolving gel. After the gel was over glass plates were removed from the

apparatus. The gel was stained either with commassie brilliant blue or silver nitrate.

4.2.5.1: Commassie Staining Of SDS-PAGE gels

Gels were dipped in the commassie brilliant blue staining solution and put on the orbital shaker for 1 hour.

4.2.5.2: Destaining of SDS-PAGE-gels

Staining solution was discarded and the gel was kept in distaining solution on an orbital shaker for 30 minutes. Distaining solution was changed 3-5 times or till the protein bands becomes clear.

4.2.5.3: Silver Staining of SDS-PAGE Gels

For detection of fine protein bands and for assessing the purity of isolated proteins the gels were subjected to silver staining. For silver staining all procedure was performed by wearing gloves.

For this, after the separation of proteins by electrophoresis the proteins were fixed by incubating for 2 hours at room temperature with gentle shaking in at least 5 gel volumes of a solution of ethanol: glacial acetic acid: water (3:1:6). The fixing solution was discarded, and 5 gel volumes of 30% ethanol were added and incubated the gel for 30 minutes at room temperature with gentle shaking. The step was repeated. Discarded the ethanol and added 10 gel volumes of deionised water. Incubated the gel for 30 minutes at room temperature with gentle shaking. This step was repeated thrice.

For staining 5 gel volumes of a freshly prepared 0.1 % solution of $AgNO_3$ was added and incubated the gel for 30 minutes at room temperature with gentle shaking.

Discarded the $AgNO_3$ solution and washed both sides of the gel under a stream of deionised water. Added 5 gel volumes of a freshly made aqueous solution of 2.5% sodium carbonate, 0.02% formaldehyde. Incubated the gel at room temperature with gentle agitation. Stained bands of protein should appear within a few minutes. Quenched the reaction by washing the gel in 1% acetic acid for a few minutes. Then washed the gel several times with deionised water.

4.3.Purification of culture filtrate proteins

4.3.1.Extraction of culture filtrate protein

M. tuberculosis $H_{37}Rv$ grown for 6 weeks was used for isolation of proteins excreted in the culture filtrate. For, this the Mycobacterial cell mass was removed by centrifugation of total growth at 15,000 g. for 30 minute at 4°C in Beckman centrifuge. The supernatant was collected in 50 ml sterile tubes and stored at –20°C till further use. After collection of sufficient amount of culture filtrate, protein obtained from different batches and harvested at the same time point the filtrate was then subjected to precipitation by ammonium sulphate. For optimization of concentration of ammonium sulphate giving maximum yield of protein the culture filtrate supernatant was subjected to precipitation by different concentration of ammonium sulphate viz. 50%, 75%, and 80% of saturated ammonium sulphate. The precipitant was kept at 2-8°C for overnight. Samples were than centrifuged at 18,000g for removal of

supernatant and collection of precipitated protein in the pellet. The yield from different precipitation method was determined by Bradford method (1976) and analyzed by SDS-PAGE.

After optimization of harvesting and precipitation conditions, the culture supernatant was finally precipitated with 80% saturated ammonium sulphate at 4°C for overnight.

The resultant precipitant was then centrifuged at 12,000 g., 18,000 g., 28000 g., and 90,000 g. for 30 minute at 4 °C in an ultra centrifuge. The pellet was collected and dissolved in 10mM Phosphate Buffer (pH-7.2). The material was dialyzed (10 KDa membrane) against the PB (10mM, pH-7.2). Buffer was changed every four hours, and was given six-seven changes of 10mM PB to ensure complete dialysis. Meanwhile, conductivity of dialysis buffer was checked on conductivity meter until it equals that of 10mM PB. The membrane was removed from the dialysis buffer and the protein was transferred to a fresh sterilized glass tube and kept at -20°C. The total protein was estimated by Bradford method (1976).

4.3.1.1.Preparation of Bradford Reagent

Bradford Reagent

Dissolved 100mg of Coomassie Brilliant Blue (CBB) G-250 (Cat no. 500-001, Bio-Red Lab) in 50ml of 95 % ethanol. Then added 100ml of 85% phosphoric Acid. Further increased the volume up to 1liter. The reagent was then filtered through Wattman No. 1 filter paper and stored at 4°C till used.

BSA Standard stock Solution (0.5mg/ml)

10 mg 0f bovine serum albumin was dissolved in sterile distilled water with smooth stirring to prevent effervescence and finally the concentration of stock solution was adjusted to 0.5mg/ml having an absorbance of 0.33 at 280 nm.

Other reagents and materials

0.15 M Sodium Chloride (NaCl), microcuvattes, and 96- well microtitre plate.

4.3.2.Protein concentration

For loading the protein on column for purification of culture filtrate proteins the total protein obtained after precipitation (100ml) was further concentrated using Amicon ultra filtration assembly having 10 and 5 k Da cut off filters. The protein was concentrated under positive nitrogen pressure at 4°C with constant stirring for 18 hours.

Finally the 5 ml concentrated volume was obtained after utrafiltration. The protein content was determined by Bradford method and stored at –20°C till further use.

4.3.3.Purification

Individual proteins from the total culture filtrate protein were purified using column chromatographic techniques. Briefly, DEAE–Sepharose CL-6B (anion exchange) was packed in the glass column having an internal diameter of 2.0 cm and 20 cm in length. The gel (DEAE-Sepharose CL-6B) was equilibrated with 10 mM Tris HCl buffer (1.214 gm Tris in 1 litre of distilled water pH 8.7) containing 3 % methylcellose. The concentrated culture filtrate proteins were dialyzed against Tris buffer and 100 mg of it was loaded on the column that

was then left undisturbed at 4°C for 30 minutes for binding of the proteins to the gel matrix. The column was eluted with equilibrating buffer and the elutant was reloaded thrice to ensure the maximum binding of protein to the column and to remove the unbound protein from the gel which was then washed with the equilibrating buffer till the absorbance of the elutant was zero.

The bound protein was then eluted from the column using 50–300mM NaCl linear gradient made in the equilibrating buffer, 125 fractions each consisting of 3ml were collected in sterile tubes. The absorbance of individual fraction was taken at 280nm on spectrophotometer using equilibrating buffer as blank and was then plotted against each fraction. The fractions of individual single peak were pooled separately in sterile 50 ml conical flask. The individual peaks were then concentrated using Amicon unit with 5kDa cut-off filter followed by dialysis against PBS overnight at 4°C to remove Tris salt. Protein content in each pooled fraction was determined by Bradford method and the protein profile was analysed by SDS-PAGE.

4.3.3.1.Purification of Individual Protein from the Pooled Fraction

The proteins in different peaks were further isolated in pure form by eluting them from the SDS–PAGE. For this preparative gel of individual peak were run as described earlier. The individual protein band was then eluted from the gel as described ahead. After completion of gel run, a thin slice of gel was cut from one side and was subjected to silver staining meanwhile the remaining unstained gel was kept in moist petri plate at 4 ^{o}C. The stained gel band was then kept adjacent to the unstained gel and individual bands matching to

single protein bands were cut with help of sharp surgical blade. The individual bands were kept in separate sterile tubes containing Tris buffer individual bands were broken into small piece and then kept overnight at 4 ^{O}C for elution of protein from the gel. Next morning the buffer containing the protein was aspirated and subjected to dialysis for removal of Tris salt. Individual sample (containing purified protein) was then subjected to SDS-PAGE for demonstration of pure bands.

4.4.Selection of immuno reactive

Using ELISA and Western blotting all the purified proteins were screened for the presence of antibodies against them in sera of confirmed tuberculosis patients.

To ensure optimal results conditions for performing ELISA was optimized by conducting the assay by varying all variable (coating buffer, pH, substrate buffer, concentration of Ags and incubation time, temperature etc.

4.4.1.Standardization of Enzyme Linked Immunosorbant Assay (ELISA)

ELISA is a very sensitive technique that measures very small quantity of antigen / antibody present in the sample. Also the assay is dependant on many variables and proper optimization of each variable is essential to obtain good results. Hence in the present study different variables of ELISA were studied and final evaluation of patient samples was done with the optimized conditions.

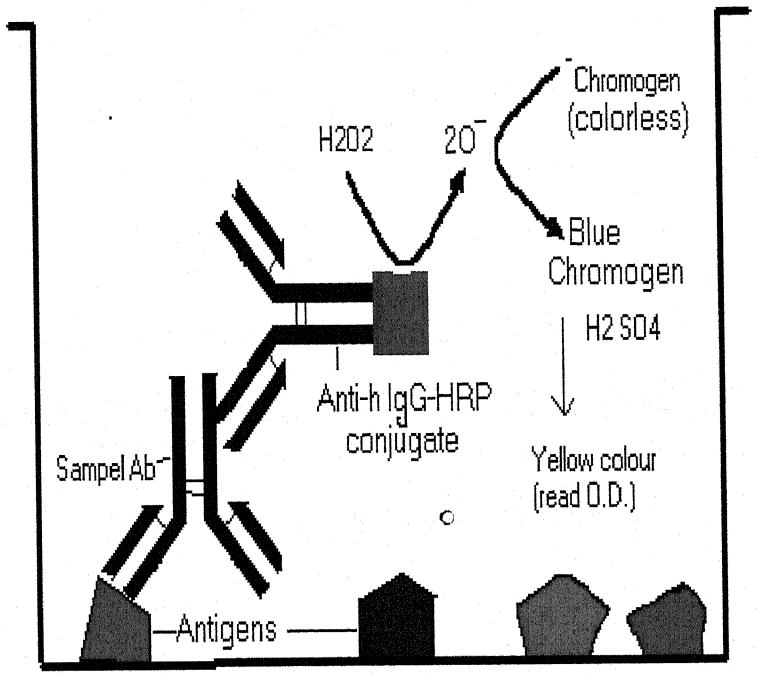


Figure 7: Schematic representation of Indirect ELISA test

Different parameters studied were

PARAMETERS STUDIED	Variable used
Coating buffer Effect of pH Ionic strength	Phosphate, Sodium carbonate / bicarbonate pH 7.2, pH 9.5 10mM, 50mM, 100mM and 200mM
Blocking agents Strength Temperature Period	Normal Rabbit serum, Bovine serum albumin & Casein 1.0 % either of three 37 ^{O}C 3 hours
Coating of antigens Concentration Volume Incubation period Temperature	 2-5µg /well 200ml 3, 5, 12, 24 hour, and 0.1, 2, 3 hour 4 and 37^{O}C
Optimization of conjugate Goat anti human IgG-HRP Time of stopping the Activity of conjugate	Ratio of OD at 450 nm at 02-03 05,10,15,20,25,30 minutes

a) Coating buffer (Phosphate buffer, 10mM, and pH-7.2):

Ingredient	**Quantity**
Di-sodium phosphate	1.70 g
Sodium dihygrogen phosphate	1.20 g.
DH_2O	1000.00 ml

Buffer was prepared by dissolving the above-mentioned chemicals in 800 ml distilled water and adjusted the pH to 7.2.

b) Sodium carbonate/bicarbonate buffer 10mM, pH-9.5

Ingredient	**Quantity**
Solution A: - Sodium carbonate	10.5 g
Solution B: - Sodium bicarbonate	8.4 g
DH_2O	1000.00 ml

c) Blocking buffer:

i) It was prepared by dissolving 1 %(w/v) BSA in 900ml of coating buffer, 10mM, pH-7.2, made up the volume to 1.0 liter with distilled water.

ii) It was prepared by dissolving 1 %(w/v) Casein digest in 900ml of coating buffer, 10mM, pH-7.2, made up the volume to 1.0 litre with distilled water.

iii) It was prepared by dissolving 1 %(v/v) Normal Rabbit serum in 900ml of coating buffer, 10mM, pH-7.2, made up the volume to 1.0 litre with distilled water.

d) Washing buffer (Phosphate butter, 10mM, pH-7.2):

Dissolved the following chemicals in 800ml-distilled water. Water and adjusted the pH to 7.2,

Ingredient	**Quantity**
Di-sodium phosphate	1.70 g
Sodium di hygrogen phosphate	1.20 g
Tween 20	0.1%
DH_2O	1000.00 ml

e) Preparation of conjugate:

Dissolved 2K Goat anti human-IgG-HRP conjugate with 50% glycerol in 20ml of blocking buffer, 10mM, pH-7.2 and added the 0.1% of Tween 20.

f) Preparation of primary Antibody (Positive TB patient sera)

Positive TB sera diluted to 1:20 in 20ml blocking buffer and added the 0.1%(w/v) of Tween 20.

4.4.2.General Procedure for performing ELISA

4.4.2.1.Coating of Antigens

The protein antigen was dissolved in coating buffer (10 mM PB, pH 7.2 and carbonate / bicarbonate buffer, pH-9.5) at a concentration of 2–5 µg / well and 200 µl of this was coated on maxisorp polystyrene surface of 96 well ELISA microtitre plates and incubated at 4°C overnight. Thereafter, the unbound antigens washed from micro wells with phosphate buffer, 10mm, pH-7.2 – Tween –80 (400µl / wells).

4.4.2.2.Blocking of immobilized antigens

The unoccupied space in the wells was blocked with 400 µl of blocking (NRS/ BSA/ Casein) solution for 1 hour at 37 °C and then removed excess blocking solution by washing buffer (10 mM PB, pH-7.2) twice at room temperature. Finally, the coated plates were vacuum dried in a dessicator containing activated silica gel and kept at 4°C till use.

4.4.2.3.Addition of antibody / patient sera

TB positive serum samples from patients, positive control, negative control (primary Ab_1) were diluted 1:20 in blocking buffer (10 mM PB, pH 7.2 with 1.0% (w/v) BSA and 0.1% (w/v) Tween-20). 200 µl of these diluted sera was applied in duplicate to the wells and incubated at 37 °C for 1-3 hour. The unbound serum was then decanted and the wells of the micro titre plate were washed with washing buffer (10 mM PB, pH 7.2 with 0.1% (w/v) Tween-20) five times at room temperature.

4.4.2.4.Addition of conjugate (Secondary antibody)

Anti-human IgG-HRP (goat) / Anti Mouse IgG HRP (rabbit) conjugate at a 1:1000 dilution in dilution buffer (10mM PB, pH 7.2, 1.0% (w/v) BSA, 0.1% (w/v) Tween-20) was added in 200ml volume to each well and incubated for 2 hour at 37 °C. After the incubation wells were washed 5 times with PB-Tween-80 Buffer.

4.4.2.5.Addition of Substrate

The peroxidase conjugate reaction with primary antibody was developed by adding 200 µl of TMB (10 mg/ml in Dimethyl sulphoxide) diluted with 1:100 with 0.1 M citrate-acetate buffer, pH 5.6 containing 0.1% (w/v) of 30% H_2O_2.The plates were incubated at 35°C in dark for 30 minutes. The reaction was stopped by addition of 50ml of 5N H_2SO_4 immediately, after the color appeared in the control wells.

The plate was then kept at 4°C and read at 450 nm. All determinations were done in duplicate.

4.4.3.Western Blotting

4.4.3.1.Reagents

Electroblotting buffer 920 mm Tris / 150 m M glycine, pH 8.0

Added 14.5 g Tris base and 67 g glycine to 4.0 litres of double distilled water. Brought the pH 8.0.Added 1200 ml methanol and made up the volume to 6.0 litres with double distilled water.

Blocking buffer (5 % Casein digest, hydrolysate)

Dissolved 5 g. casein digest, hydrolysate in 100 ml phosphate buffered saline, 10 mM, pH 7.2

Wash Buffer (Phosphate buffered saline 10mM, pH 7.2)

Ingredient	Quantity
Di-sodium phosphate	1.70 g
Sodium di hygrogen phosphate	1.20 g
Tween 20	0.1%
DH_2O	1000.00 ml

Primary antibody

1:50 Tuberculosis patient sera was used

Secondary antibody (anti human IgG - Hoseradish Peroxidase conjugate)

1: 2.5 k strength of the conjugate was used.

Diaminobenzidine (DAB) substrate solution

Ingredient	**Quantity**
3, 3' – diaminobenzidine	50 mg
1 % Ni Cl_2	2 ml
Phosphate buffered saline, 10 mM, ph 7.2	98 ml
30 % H_2O_2	0.1ml

Selection of immunoreative protein among the purified proteins of *M. tuberculosis* was also made by western blotting by evaluating the ability of these proteins to be detected by the antibodies present in the sera of confirmed tuberculosis patients.

4.4.3.2.Procedure

Western blot of the purified proteins was performed as per standard protocol. Briefly, the purified proteins were run on 12 &15% SDS-PAGE and then the electrophoresed proteins were transferred to nitrocellulose membrane by trans-blotting. Nitrocellulose membrane was equilibrated with transfer buffer and kept over filter paper stack carefully, removing all the air bubbles. The SDS gel was then gently kept over the nitrocellulose paper. Another three sheets of filter paper were added on the top of the gel taking care that no air bubble was there. Blotting was conducted under constant electric current of 100 mA for 2 hours to ensure all the protein bands are transferred.

Finally, the blots were washed with PBS and soaked for 5-10 min in 0.2% of Ponceau S solution in PBS and incubated at room temperature with gentle

agitation. Transferred the nitrocellulose sheet to PBS and rinsed for 2min with several changes of PBS. Marked the position of transferred protein bands. If distinct bands were apparent, the membrane was blocked in blocking buffer (PBS with 0.1% Tween-20 and 5% Casein digest) for overnight at 4°C over an orbital shaker and then incubated in 10 ml 1:50 dilution of primary antibody / serum in dilution buffer (PBS with 0.1% Tween-20) containing 5% Casein digest for 2 hours at room temperature. At the end of incubation, the membrane was washed five times for 5 minutes each by agitating over an orbital shaker with 50 ml. of PBS with 0.1% Tween-20. HRPO-anti-human IgG conjugate (Dako, USA) was diluted 1:2500 in dilution buffer (PBS with 0.1% Tween-20) containing 5% Casein digest and membrane was incubated for 2 hour at room temperature with constant agitation. At the end of incubation, the membrane was washed five times for 5 minutes each with 50 ml. of washing buffer and finally washed in 10 ml of 10 mM Tris-Cl (pH-7.5) for 5 minutes. The blots were then developed with 0.8 mM DAB (diaminobenzidine hydrochloride) (Sigma, USA) in the presence of 0.045% H_2O_2 and 0.4 mM Nickel Chloride for 10-20 minutes till clear bands were visible. The membrane was kept in PBS to stop the reaction.

4.5.Production of Polyclonal antibody to culture filtrate protein

Polyclonal antibodies were raised in rabbit against total culture filtrate proteins (CFP) of *M. tuberculosis* strain $H_{37}Rv$ as under.

4.5.1.Preparation of antigen – IFA complex

Antigen mixture for immunization was prepared as follows: 1mg of protein in PBS (pH 7.5) was emulsified thoroughly with incomplete Freund's adjuvant (IFA). The double hubbed needle method as suggested by Berlin and McKinney in 1968 was used for making immunogen preparation. The immunogen was prepared by mixing equal volume of reactive immunogen with incomplete Freund's adjuvant (IFA). The emulsion was tested before use by putting one drop of emulsion over water. It remained intact, thereby, confirming that emulsion was ready for use.

4.5.2.Immunization of Rabbits

Five rabbits (6 months old) were immunized by subcutaneous (500µl/Rabbit) injection. All animals were given booster on day 14 and 28 in IFA to enhance the antibody titer. The rabbits were bled after 14days after the last dose using a fine capillary tube. The orbital conjunctiva was ruptured with controlled hand pressure and blood was collected in a fresh tube and kept as such at room temperature serum was separated and subjected to purification.

4.5.4.Purification of Antibodies

Rabbit anti serum (5 ml) diluted to 50 ml in Tris-Hcl (0.1 M pH 8.2) was loaded on pre-packed protein A sepharose column XK16/20 (Amersham Pharmacia Biotech, Sweden) equilibrated with Tris-Hcl buffer (pH-8.2). Unbound proteins were washed with Tris-Hcl and the antibody bound to the affinity column was eluted with 0.1 M Glycine-HCl buffer (pH 2.5) at a flow rate of 1 ml/minute into 4 ml fractions (Figure-8). The pH of the eluted fractions was adjusted to 7.2

using saturated Tris-Hcl buffer (pH 9.6). The protein content and purity of each fraction was monitored at 280 nm and 10% SDS-PAGE, respectively. Fractions containing purified antibody (IgG) were pooled, dialyzed against PBS buffer (pH 7.5) for 24 hours with two buffer changes at equal time intervals. Concentration of the protein was estimated using Bradford method. Finally, the antibody solution was lyophilized and stored at 4°C for further use.

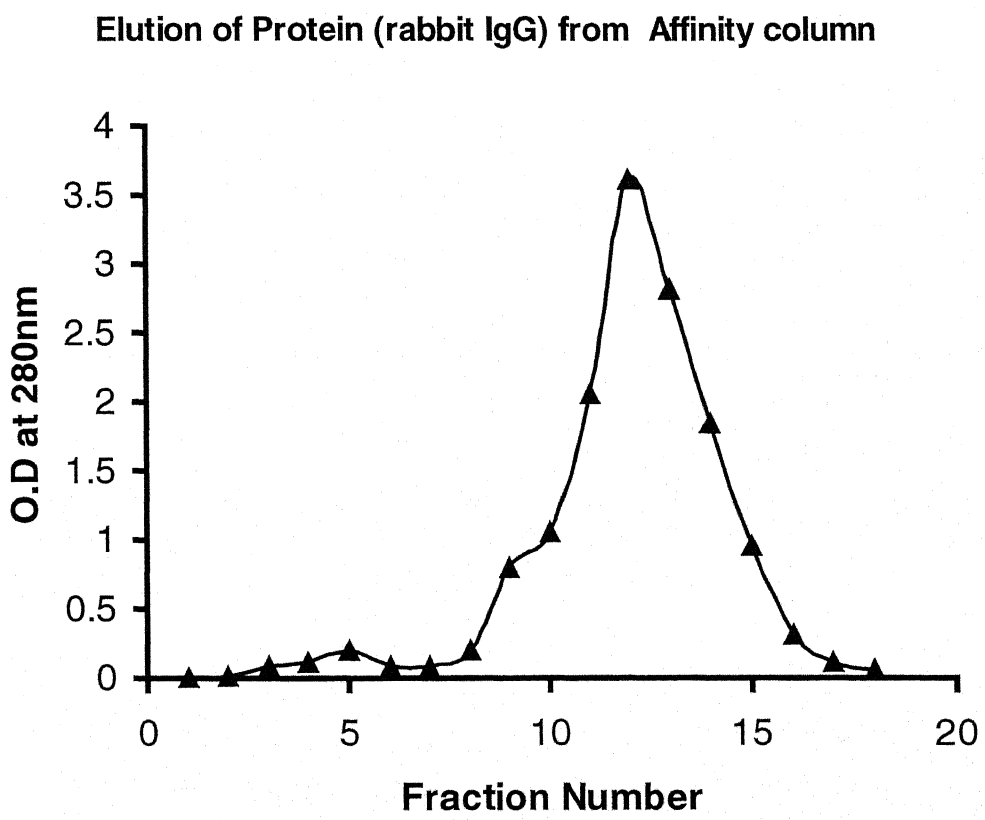


Figure 8: Elution profile of Ig G antibody from the affinity column

4.6.Immunogenicity and immunoprotective studies

All the purified secretory proteins were tested for their ability to: -

- Induce significant humoral response for its use in sero diagnosis.
- Induce significant cellular immune response for its possible role in inducing protective immunity against *M. tuberculosis* infection.

4.6.1.Selection of antigen(s)

All the purified secretory proteins were screened for their immunoreactivity in mice as detailed below.

Preparation of protein FIA complex

For immunization, proteins (total culture filtrate protein) in phosphate buffer were mixed with equal amounts of incomplete Freund's adjuvant (IFA). The water in oil emulsion was prepared by mixing the two phases until a thick emulsion formed. The resulting emulsion was then tested for its stability by placing a drop of it on to the surface of cold water and observing for the tendency of the drop to stay intact. The intactness of the drop on the water indicates that the emulsion is ready to use.

Immunization Protocol

Animal was immunized subcutaneously on day 0,7 and 14 with the antigen complexed with IFA. The immunizing dose for total culture filtrate protein was 100 mg / mice split in three dose i. e. 33.3 µg / dose. Following group (each consisting of 20 mice) were included to study the immunoreactivity of isolated secretory proteins.

Group	Immunized with
I	Mice immunized with total culture filtrate protein (CFP-IFA)
II	Mice immunized with live *M. tuberculosis* $H_{37}Rv$ cells ($1x10^6$ cells / animal)
III	Mice immunized with normal saline (NS-IFIA)

Immune Response (S)

In the above-mentioned group both cellular and humoral immune responses were studied at weekly, post immunization intervals by ELISA (as described earlier), T-lymphocyte proliferation assay described later (Section 4.10.1). The pre and post immune sera from animal were obtained by bleeding from retro-orbital Venus plexus.

4.7.Immunigenicity of most immunoreactive secretory protein (MISP) - in IFA

4.7.1 Preparation of Antigen

Different concentration of most immunoreactive secretory protein (MISP) identified on from the above study was evaluated for its ability to induce immune (humoral and cellular) response. For this, three serial two-fold concentration i. e. 18.75 µg, 37.5 µg, and 75 µg were chosen for immunization to study the induction of immune response. The antigen was mixed with equal volume of IFA (1:1) to obtain stable water in oil emulsion.

Immunization Protocol

Four groups (20 mice each) were subjected to the following treatments.

Group	Immunized with
I	Mice immunized with 18.75 µg concentration of MISP in FIA
II	Mice immunized with 37.5 µg concentration of MISP in FIA
III	Mice immunized with 75.0 µg concentration of MISPin FIA
IV	Control group immunized with normal saline in IFA

The antigen was administered in three equally dose on day 0,7 and 14 by subcutaneous route.

Measurement of Immune Responses

The immune response to purified antigen was measured at weekly post immunization intervals. Animal (4-5) from each group were bled for humoral response as studied by ELISA and sacrificed for cell mediated immune response studied by T-lymphocyte proliferation assay.

Comparison of Immune response of most immunoreactive secretory protein (MISP) with the BCG Vaccine

The immune response induced by the optimal concentration of MISP was compared with the responses in BCG vaccinated animals. For this, three groups of animals, consisting of 20 mice each were immunized with respective immunogen as given below: -

Group	Immunized with
I	Immunized with MISP in IFA in three doses on day 0,7& 14.
II	Vaccinated with live BCG vaccine $1x10^6$ cfu's/mouse intravenously
III	Control group immunized with normal saline-FIA

Measurement of the Immune Response

The cellular and humoral activity in the above-mentioned group was determined till the fourth week p. im. The immune responses were measured by ELISA, T-lymphocyte proliferation assay.

4.8.Immunoprotective Activity of MISP against Experimental Tuberculosis

To study the immunoprophylactic properties of MISP against experimental tuberculosis, animal were immunized as given below:

Group	Immunized with
I	Immunization with (MISP)-FIA (75mg concentration of antigen)
II	Immunization with (MISP)-FIA (37.5mg concentration of antigen)
III	Immunization with (MISP)-FIA (18.75mg concentration of antigen)
IV	Vaccination with BCG vaccine
V	Control group immunized with normal saline-FIA

The antigen was administered in three equally dose on 0, 7 and 14days by sub cutaneous route. Three weeks (21days) after complete immunization, mice were challenged intravenously with LD_{50} (3×10^7 CFU's / mouse) *M. tuberculosis* $H_{37}Rv$ and the following parameters were used to assess the

protection provided by immunization with different concentration of cocktail of secretory protein.

4.8.1.Percent Mortality / Survival

Survival rate (alive *Vs* total) in immunized as well as control mice were observed up to 30 days post challenge (p. c.) and the data was evaluated using Chi square test.

Enumeration of viable bacilli from infected organs

Immunized / control mice infected with *M. tuberculosis* H_{37}Rv were sacrificed in a group of 3-5 by cervical dislocation on day 30 post challenge. Lungs, Liver and spleen from these mice were removed aseptically and viable bacilli load was determined, according to the gernal laboratory method.

4.9.Immunoreactivity of *Mycobacterial* MISP secretory protein in Liposomes

4.9.1.Preparation of liposomes

Multilamellar liposomes were prepared by freeze thaw method as described by Mayer *et al.,* (1986). Briefly, 56 mg of egg phosphatidyl-choline (PC), 8.0 mg phosphotidylglycerol (PG) and 16mg cholesterol (CH) were dissolved in 100ml of chloroform: methanol, 2:1 (v/v) in a round bottom flask. The chloroform: methanol was evaporated in a rotory vacuum evaporator at 37 °C. The residual chloroform: methanol was removed by dessication overnight. Thin film of lipid that is formed on the inner walls of the flask was dispersed by gentle shaking for 2 hours in 15 ml sterile PBS (pH7.2). Thereafter, the milky suspension was distributed into 1.5ml capacity eppendroff tubes and freezed the same by immersing in liquid nitrogen and immediately thawing the frozen

preparation at 35°C. This cycle of freeze thaw was repeated five times and the liposomes were then centrifuged at 100,000g for 1 hour in an ultra centrifuge to remove the un-entrapped material. The pellet containing the liposome preparation was washed thrice with PBS (pH 7.2) and finally suspended in the same. The multilamellar character of liposome preparation was checked under microscope.

4.9.2.Preparation of antigen containing liposomes.

The basic method for the preparation for antigen containing liposomes was the same as described above for the empty liposomes except that PBS in the initial step contained 5-10 mg of MISP and was added to the thin lipid film in the flask. At the end, the liposomal preparation containing entrapped antigen was centrifuged twice in PBS (pH. 2). The percent entrapment of the antigen in the liposome was calculated by estimating the protein in the pellet and the supernatant by the method of Lees and Paxman (1972), modified version of Lowry's method.

$$\text{Percent entrapment} = \frac{\text{Protein in pellet}}{\text{Sum of the protein in pellet+ Supernatant+ washings}} \times 100$$

The resultant liposomal preparation [freeze-thaw multilamellar vesicles (FTMLV)] with or without antigen was kept at 4 °C till use.

Immunization protocol

To study the effect of entrapped-in liposomes to induce immune response was studied. For this, mice were immunized subcutaneously on days 0,7 and

14 with 75 µg of antigen (divided in three equal doses) entrapped in liposomes. Following groups were included in the study.

Group	Immunized with
I	Immunized with MISP in liposomes in three doses on day 0,7& 14.
II	Control group immunized with normal saline in liposomes.

The immune response both (cellular and humoral) were studied at different time interval after immunization by ELISA and T-lymphocyte proliferation assay as explained in section 4.4 to 4.4.2.5 and 4.10.1.

Protection studies

Group of mice immunized with MISP entrapped in liposomes was challenged with LD_{50} (3 x107 CFUs) dose of *M. tuberculosis* $H_{37}Rv$, 8 weeks after the complete immunization. The protection afforded by immunization with MISP-Lip was assessed by survival rate in immunized / control animals till 30 days post challenge and by enumeration of viable counts in the infected organs lung, liver and spleen on day 30 post challenge. Described in section 4.10.3.

4.10.Study of cell mediated immune responses

Cell mediated response to purified secretory proteins by measuring the T-cell proliferation induced by these antigens.

4.10.1.T-lymphocyte proliferation assay

This assay was performed as described by Anderson *et al.,* (1991) Immunized / control animals in groups of 3-5 each were used at each dose levels and

time interval. Mice were sacrificed by cervical dislocation and spleen of the animals were removed aseptically and washed in sterile saline to remove the surface contaminants. The spleens were then profused with RPMI 1640 medium using 10 ml syringe having 26 G needles to obtain the single cell suspension of splenocytes. The cell suspension was then centrifuged twice at 2000 rpm for 10 minute in RPMI 1640 to remove the unwanted soluble contaminants. The RBC's in the suspension were lysed by suspending the cell pellet in 0.9% NH_4Cl at 37 °C for 5 minutes. The suspension was then centrifuged twice at 2000 rpm to completely remove lysed RBC and NH_4Cl. Macrophages were then removed by pouring the cell suspension in to the glass petri plate and incubated at 37°C for 45 minutes in 5-10% CO2.Supernatant containing T and B-lymphocyte was collected with sterile syringe and centrifuged. B-lymphocytes were removed from the cell suspension by panning. For this one day prior to experiment a plastic petriplate was coated with anti mouse Ig at 4°C overnight. The cell suspension containing T and B lymphocyte was poured in the petriplate and incubated at 37°C for 30 minutes in 5-10% CO_2 atmosphere. The supernatant coating majority of T lymphocytes was collected and centrifuged twice. Finally, the cell pellet was suspended in RPMI-1640 containing 10% FCS. The cells were then counted on the hematocytometer and the cell concentration was adjusted to 2 x 10^6 cells per ml. The macrophages were removed from the petriplate by scraping with the help of rubber policeman and collected in separate tube containing RPMI-1640 and stored at 4°C.

The lymphocytes (100 µl each of 2X 10^6 cells/ml) were dispensed in the wells of 96 well microtitre tissue culture plate (Corning, USA). The T-lymphocytes of immunized and control animals were sensitized with the appropriate concentration of antigen diluted in RPMI 1640 with 10% FCS. Tissue culture plate was then sealed with sterile sealing tape and incubated at 37°C 5-10% CO_2 atmosphere for 18 hours.

The cells were harvested on the glass fibre filter using microplate harvester (Nunc). The incorporated radioactivity was counted on a β–liquid scintillation counter. The proliferation was expressed as stimulation index calculated as

Stimulation Index = Mean CPM of stimuted cells/ Mean CPM of unstimulated cells.

4.10.2.Determination of LD_{50} of *M. tuberculosis* in Mice

LD_{50} of *M. tuberculosis,* $H_{37}Rv$ injected intravenously was determined according to the methods described by Karber (1931). *M. tuberculosis* $H_{37}Rv$ cells were grown in modified Souton's medium 0.05% Tweem-80 for 8 days continuous shaking at 120 rpm at 37°C. The cells were harvested on 8^{th} day by centrifuging the culture at 6000 rpm for 15 minutes. The organisms were washed twice with sterile saline (0.85%) and suspended to the approximate concentration of 10^9 viable bacteria per ml (using Mc Farland's standards). Ten fold serial dilution of this suspension was made in sterile normal saline. Five groups of mice (10-15 each) were injected intravenously with 100 µl of appropriate suspension of *Mycobacteria* per animals.

Mortality rate in infected animals was monitored for a period of thirty days, at the end of which LD_{50} was calculated according to the methods described by Karber (1931).

$$LD_{50} = 0.5 + \text{Log}_{10} \text{ of highest concentration of bacterial dilution} - \frac{\% \text{ mortality}}{100}$$

4.10.3.Enumeration of Viable Organisms in Infected Organs

Immunized / control animals infected with *M. tuberculosis* $H_{37}Rv$ were sacrificed in group of 5 on day 30 post challenge. Different organs *viz.* spleen, liver and lung were removed from each animal aseptically. Each organ was homogenized in 3 ml of sterile normal saline in a glass homogeniser. Serialdilution (1:100 onwards) of this homogenate were done and cells were accordingly plated on solidified souton's medium supplemented with 1% BSA and colony forming units were counted after incubation at 37 °C for 4 weeks. To differentiate the growth of *M. tuberculosis* from *M. bovis* BCG, 1mg/ml of 2-thiophene-carboxylic acid hydrazide (Orme & Collins, 1983) was supplemented to the above medium. This compound selectively inhibits the growth of *M. bovis* BCG but does not affect that of *M. tuberculosis*.

5

Results

5.1. Morphological and Cultural Identification of Culture Used In the Study

M. tuberculosis $H_{37}Rv$ ATCC-27294 included in the study were grown and maintained on L. J. media slants and as glycerol stocks kept at –70°C.

Both the strains were checked for typical *Mycobacterium* morphology. For this both the cultures were stained with Gram's staining and acid fast (ZN) staining and observed under microscope (100x magnification).

Culture was not stained by gram staining procedure and appeared as unstained objects. However, when stained with acid fast staining by Zeihl-Nelson method they were seen as dark pink slender pleomorphic rods (Figure- 9).

On LJ (Lowenstein–Jenson) medium slants both the cultures grew after 28 days of incubation at 37°C and appeared as dry, rough, irregular and buff colored colonies (Figure -10).

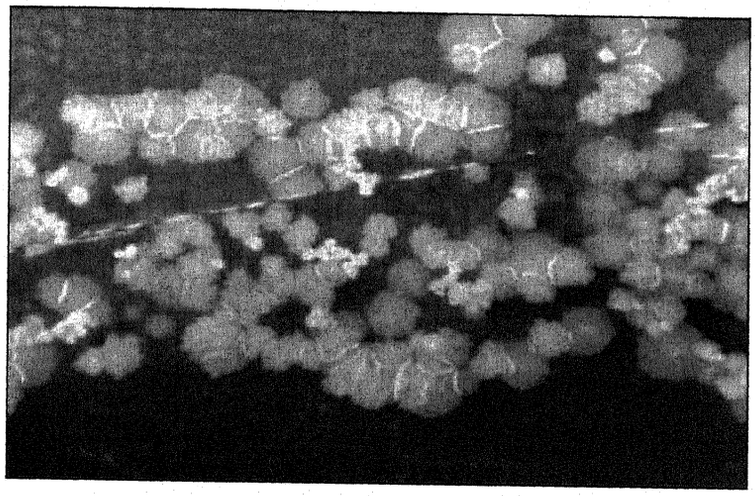
In liquid (Sautons, Middlebrook 7H9) media growth (turbidity) was visible from day 14 on the surface of flask kept under stationary condition small pellicle growth was observed which kept increasing thereafter and covered the entire top surface of media in the 500 ml flask containing 250 ml medium by the end 7^{th} week.

The purity of the culture was checked by performing acid fast staining. Growth curve was plotted by taking the absorption of culture at 580nm once every week (Figure -11) for 7 weeks. The culture was in lag phase from day 1 to

21day after which the culture multiplied exponentially (as evident for sharp increase in OD values) upto day 40.Thereafter culture entered the stationary phase till 60th day and then a decrease in the OD was seen indicating the onset of decline phase.

Both the *Mycobacterial* cultures were also tested for the typical *M. tuberculosis* character and were found positive for nitrate reduction and niacin test confirming them as *M. tuberclosis.*

a



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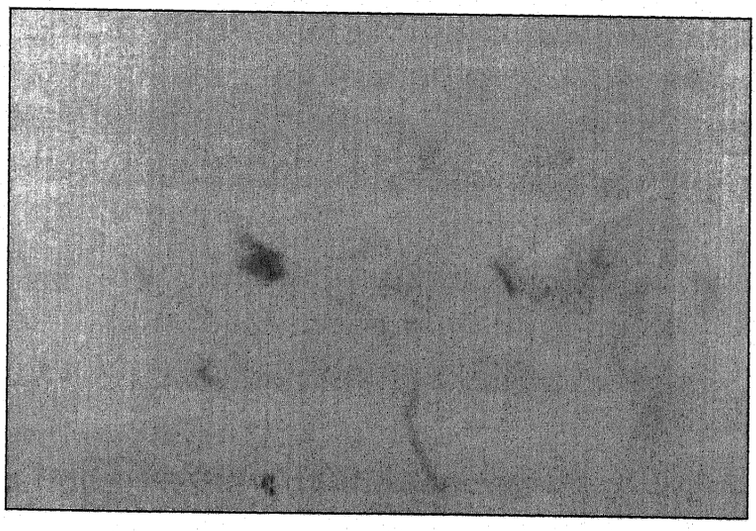

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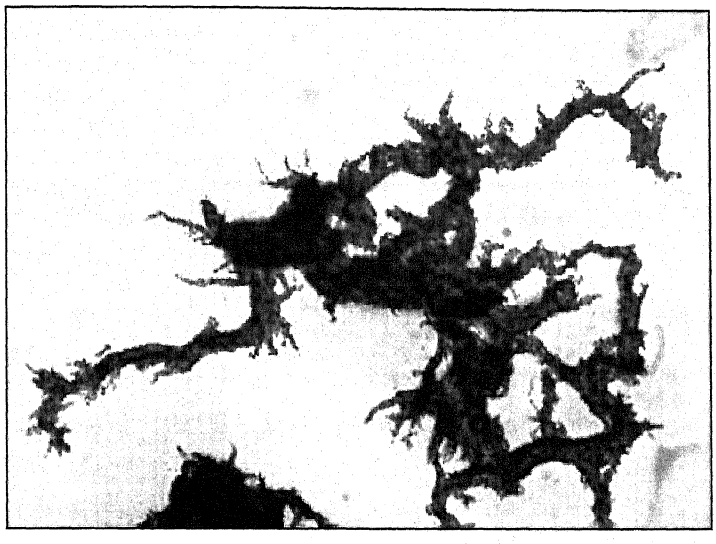

Figure 9: Growth of *M. tuberculosis* culture on (a) solid medium and (b, c) on liquid medium

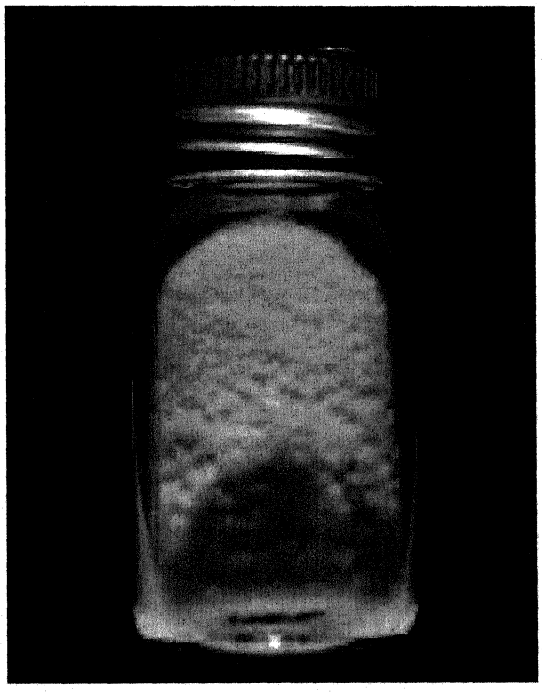

Figure 10: Growth of *M. tuberculosis* on LJ medium

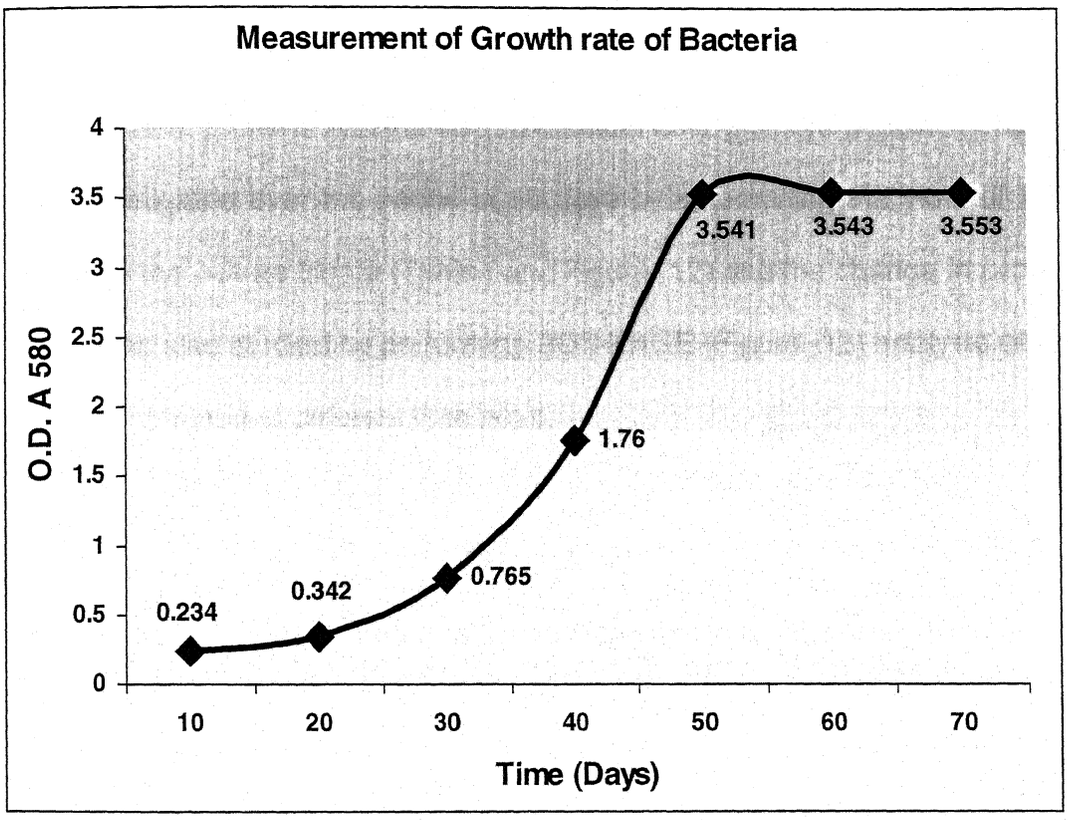

Figure 11: Growth curve of *M. tuberculosis* $H_{37}Rv$ on Sauton's medium at 37°C

Profile of proteins released in culture medium during growth

It is well known that during growth of microorganism it releases various substances in to the medium. These substances can be metabolic by products, Lipids, carbohydrates and proteins. Proteins released into the mediums, which are secreted by actively growing cells, and those released following the lyses of bacterial cell into their surrounding medium. Therefore the release of proteins into the culture medium during different growth phases was investigated over the period of 50 days by determining the yield of total protein in the culture filtrate (Table-I and Figure- 12) and the change in protein profile was also studied by performing SDS-PAGE (Figure -13) analysis of the samples obtained at different time point.

Table - I

Protein released in the medium during growth on M. tuberculosis in Souton's Medium collected at different time points						
1 week	**2 week**	**3 week**	**4 week**	**5week**	**6week**	**7week**
0.231	0.321	0.881	1.73	3.21	3.3	3.32

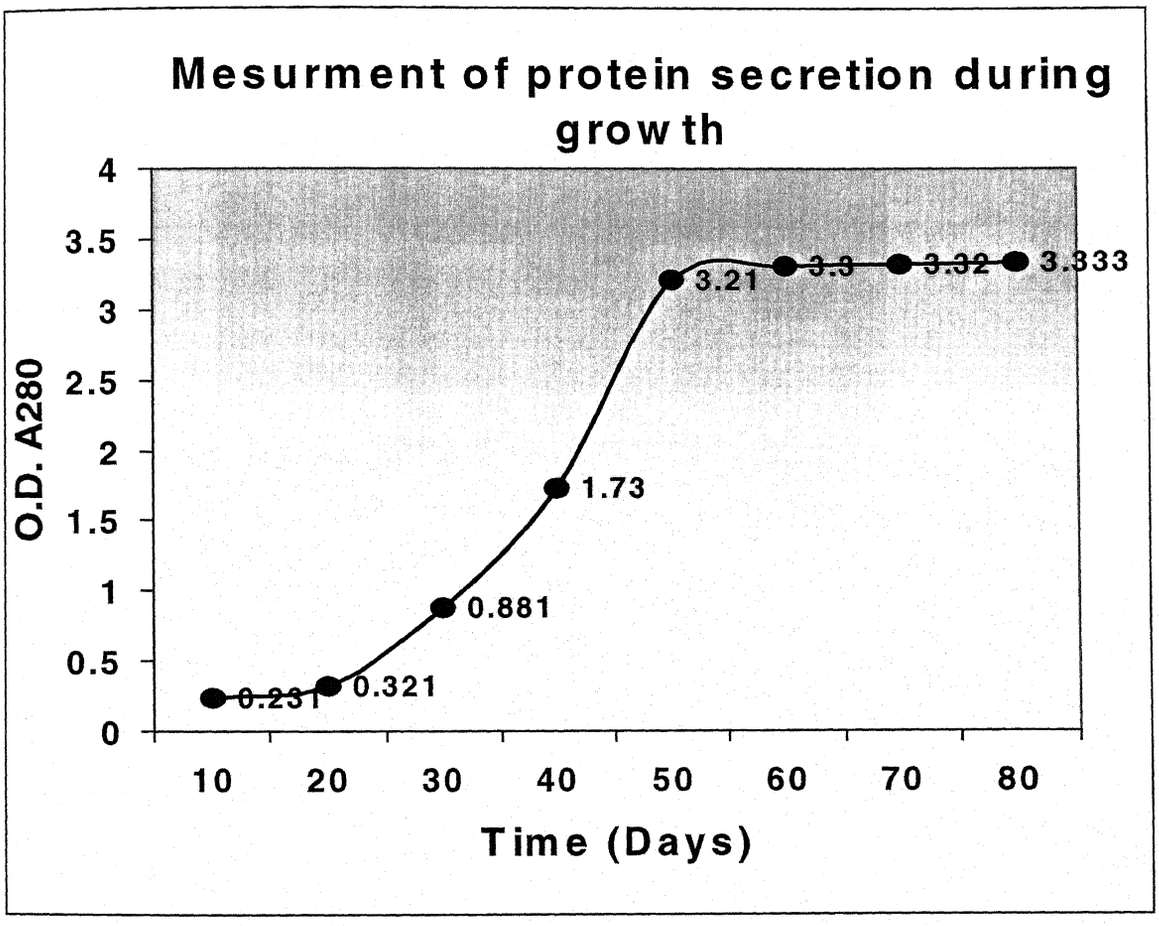

Figure 12: Secretion of protein into the culture medium during the growth of *M. tuberculosis* $H_{37}Rv$ in culture liquid under stationary condition.

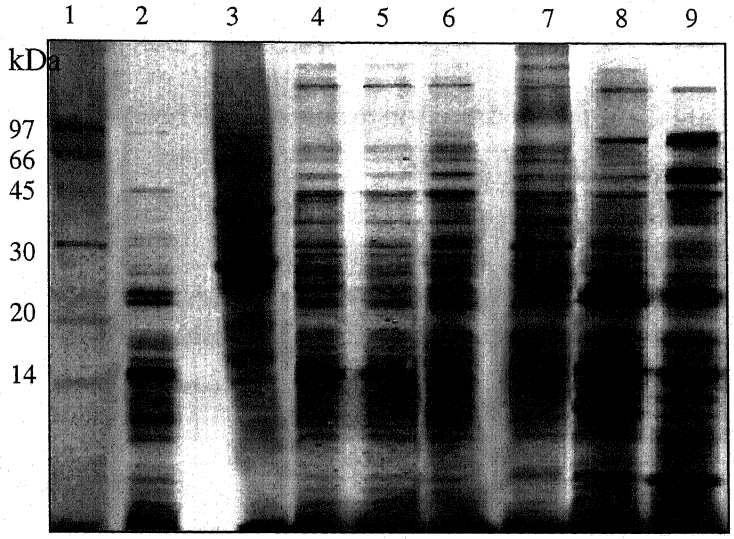

Figure 13: Photograph showing Secretion of protein into the culture medium during the growth of *M. tuberculosis* $H_{37}Rv$ in culture liquid under stationary condition form SDS PAGE Silver staining.

Purification of major proteins from the culture filtrate proteins

Culture filtrate of mid log phase growth was fractionated on DEAE Sepharose CL 6B column and its elution profile is shown in Figure-14.The total culture filtrate protein was resolved in to six peaks when eluted with NaCl gradient. Fractions under six peaks were pooled and concentrated by ultrafiltration. The peaks were designated as FI, FII, FIII, FIV, FV, FVI, respectively. The amount of protein in six peaks is shown in Table-II, where approximately 83.24% of the total CFP's applied recovered as unbound and from different peaks.

Table- II
Amount of protein recovered from different peaks of total culture filtrate protein after DEAE Sepharose CL 6B column

Conc. Of Protein	Elution Peaks						
	Unbound volume	FI	FII	FIII	FIV	FV	FVI
Total Protein	17.16	13.8	10.69	6.5	7.8	8.3	14.67
%age recovery *	20.38	14.5	11.25	6.8	8.2	8.7	15.44

* Total culture filtrate protein loaded on to the column was 95 mg

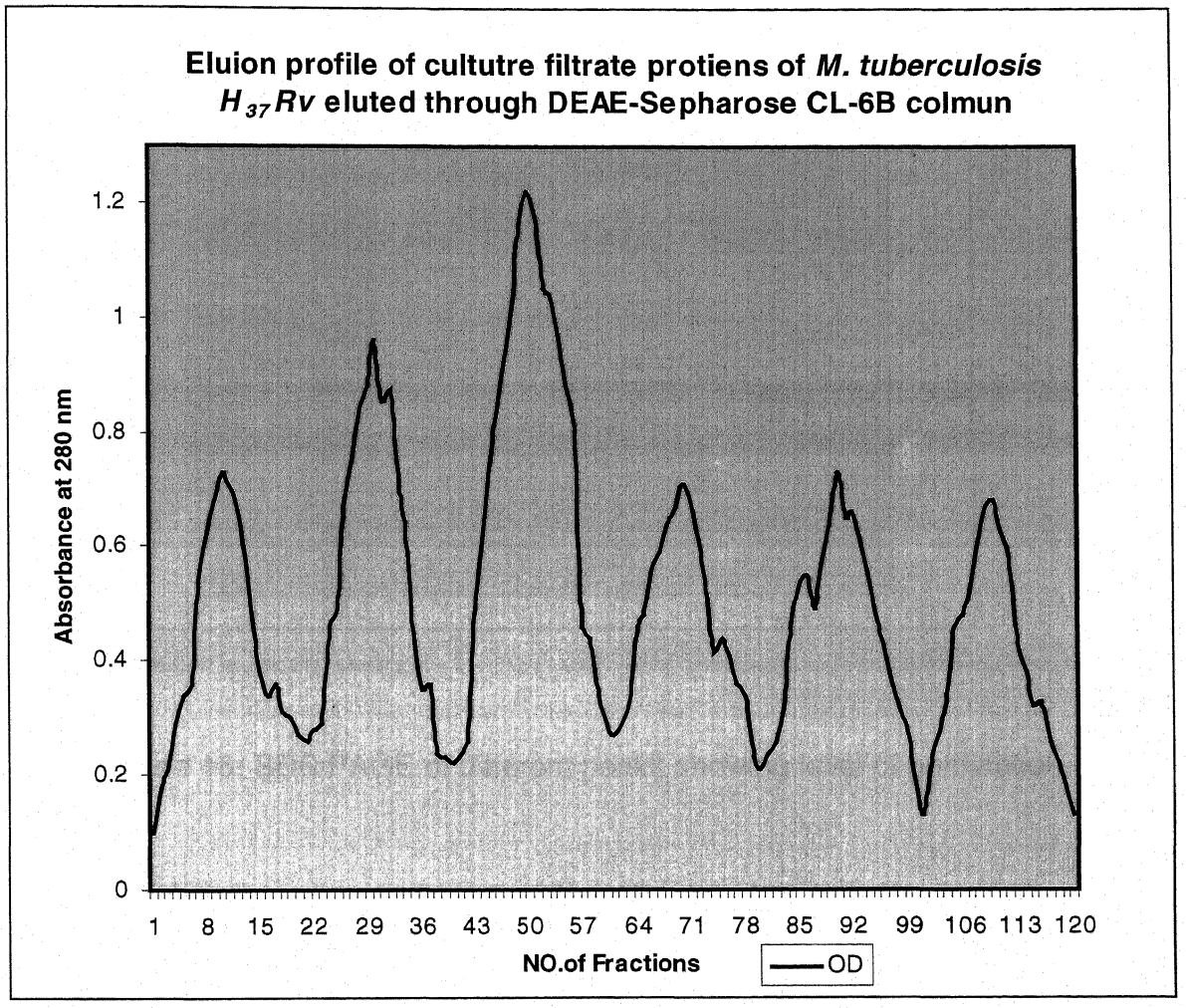

Figure 14: Column elution profile of Culture Filtrate Protein

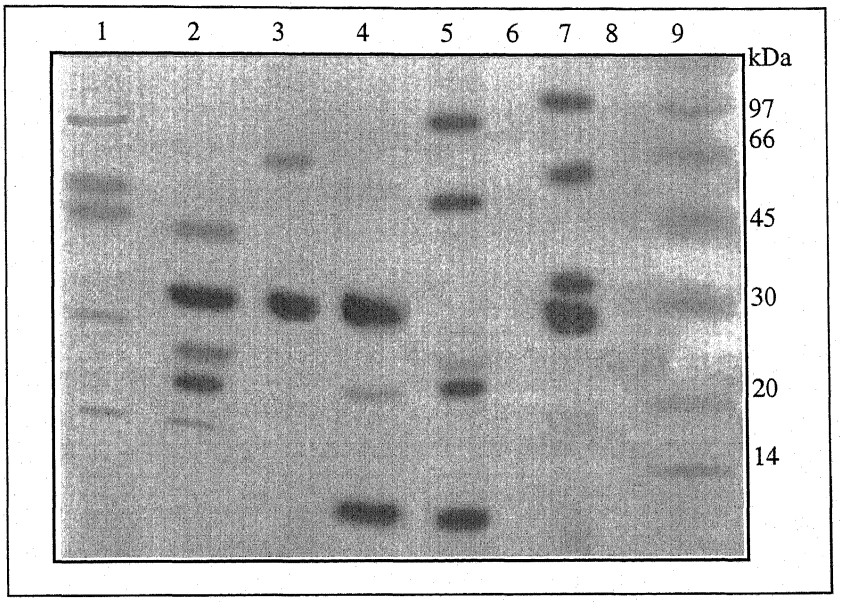

Figure 15: SDS PAGE of different peaks showing level of purification

The peaks were analyzed by SDS-PAGE to examine the separation of proteins in different peaks (Figure-15). Peak III yielded pure 30kDa protein, the other fraction contained more than 3-4 proteins. These proteins were further purified using elution method from SDS-PAGE gel in Tris Buffer as explained in materials and method. Four different proteins were purified by elution method. Thus, a total of five different proteins were purified from the total culture filtrate protein.

The molecular weight of all the six proteins were determined using SDS-PAGE by comparing relative mobility of the prestained standard molecular weight markers, which yielded molecular weight of 6kDa, 26kDa, 30kDa, 38kDa, and 64kDa respectively (Figure -16 and 17).

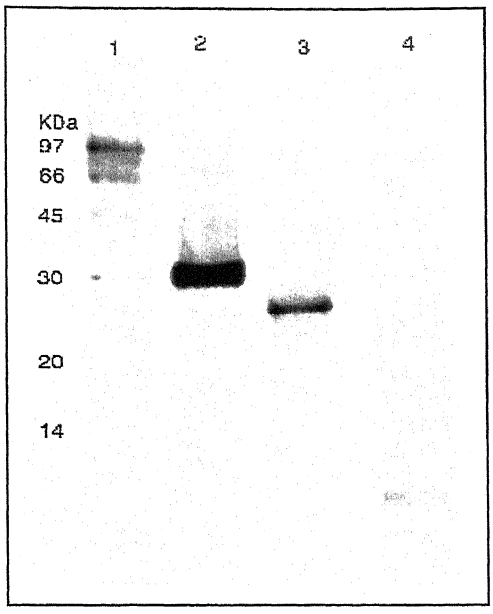

Figure 16: Photograph showing three purified proteins from SDS PAGE run on 12 % gel

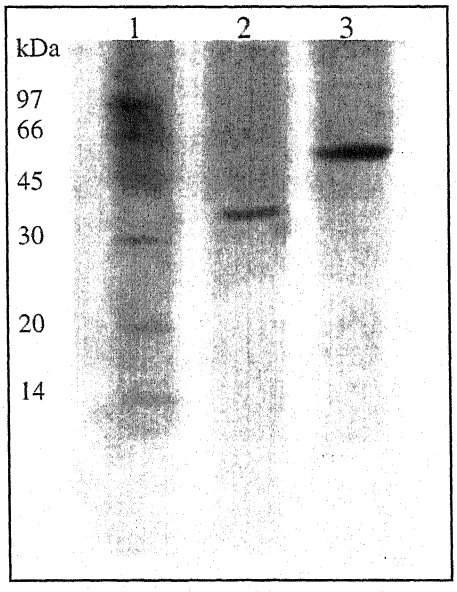

Figure 17: Photograph showing two (64 and38 kDa) purified proteins from SDS PAGE run on 12 % gel

Selection of most immunoreactive antigen using tuberculosis Patient Sera

After the purification of the five secretory proteins from the total culture filtrate, the purified proteins were then reacted with sera of confirmed tuberculosis patients in western blot for the selection of antigen(s) having antibodies in patient sera. From the results of western blot (Figure- 18 to 23) it is evident that of the five antigens antibodies against two-protein viz. 64 and 26 kDa were present in significant amount in sera of all patients tested. The antibodies to other antigens were also seen in the sera of patients but not in all patients tested and in some it was present in low amount (as indicated by fine bands). In an attempt to develop diagnostic kit by performing the ELISA for diagnosing the TB patient's sera a cocktail of purified antigens was also used in addition to individual antigens.

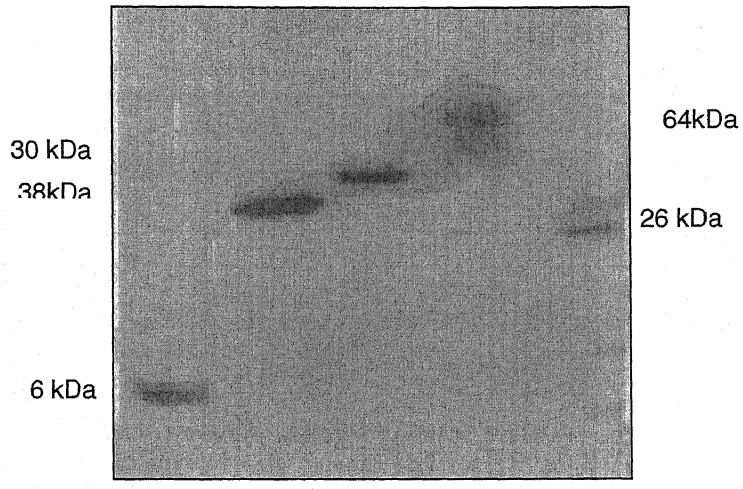

Figure 18: Photograph showing immunoreactivity of five purified Secretory proteins with tuberculosis patient's sera from western blot.

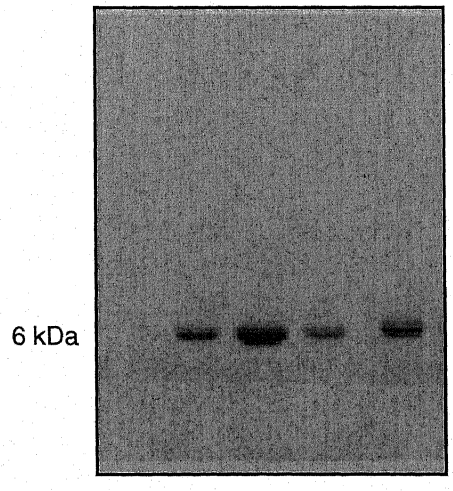

Figure 19: Photograph showing immunoreactivity of 6 kDa purified Secretory proteins with extra pulmonary tuberculosis patient's sera from western blot.

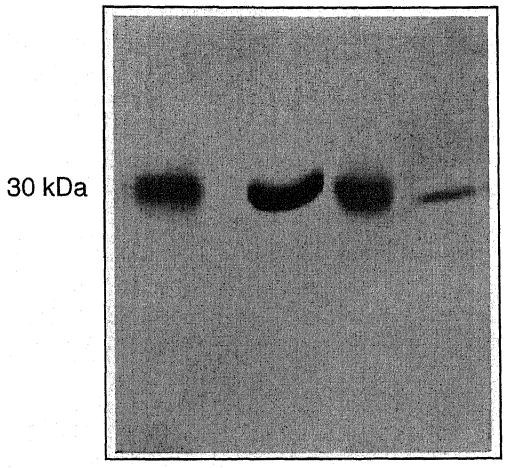

Figure 20: Photograph showing immunoreactivity of 30 kDa purified Secretory proteins with extra pulmonary tuberculosis patient's sera from western blot.

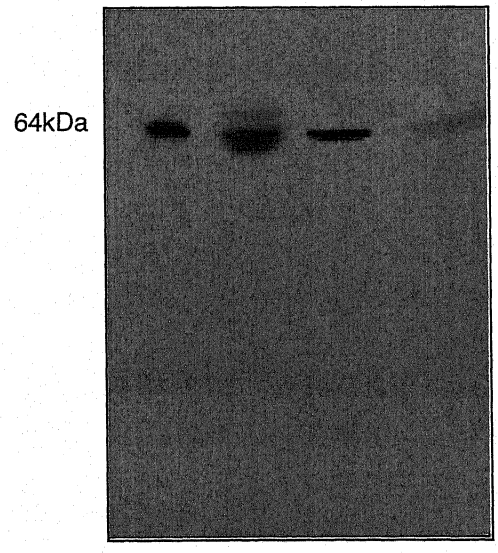

Figure 21: Photograph showing immunoreactivity of 64 kDa purified Secretory proteins with pulmonary tuberculosis patient's sera from western blot.

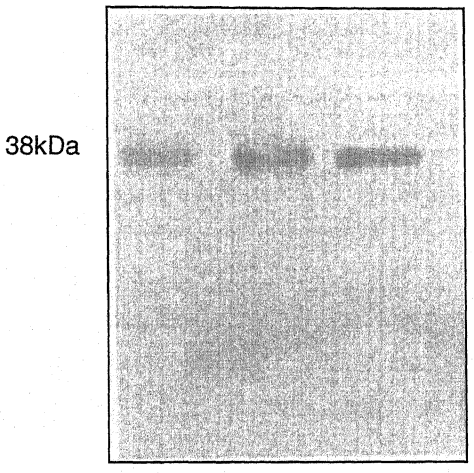

Figure 22: Photograph showing immunoreactivity of 38 kDa purified Secretory proteins with pulmonary tuberculosis patient's sera from western blot

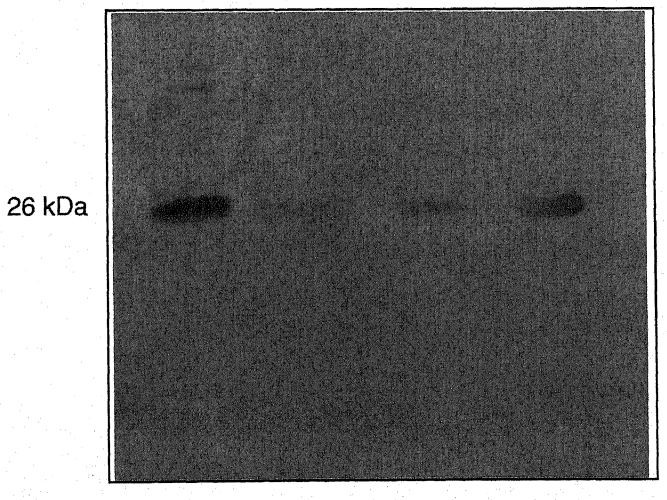

Figure 23: Photograph showing immunoreactivity of 26 kDa purified Secretory proteins with pulmonary tuberculosis patient's sera from western blot.

Standardization of ELISA

The optimal conditions for performing ELISA to detect the presence of antibody in the sera of human tuberculosis patients and to measure the Humoral response in animals to the purified antigen(s) were standardized for Secretory antigens.

Effect of blocking agents

To enhance the specificity, various types of blocking protein were tried such as BSA, Casein digest, Normal Rabbit serum were used to eliminate non-specificity. Among all the mentioned blocking agents tested, Casein digest was found to be the best blocking agent which gave close to zero noise level, hence 100% specificity. (Table – III and Figure -24). The implementation of Casein digest at a concentration of 1% helped in achieving 100 % specificity along with process feasibility.

Table - III

Effect of blocking agents on the specificity / reactivity of antigen with antibody

Blocking agent	O. D. (450nm)
BSA	0.060-0.350
Casein digest	0.020-0.150
Normal Rabbit sera	0.022-0.250

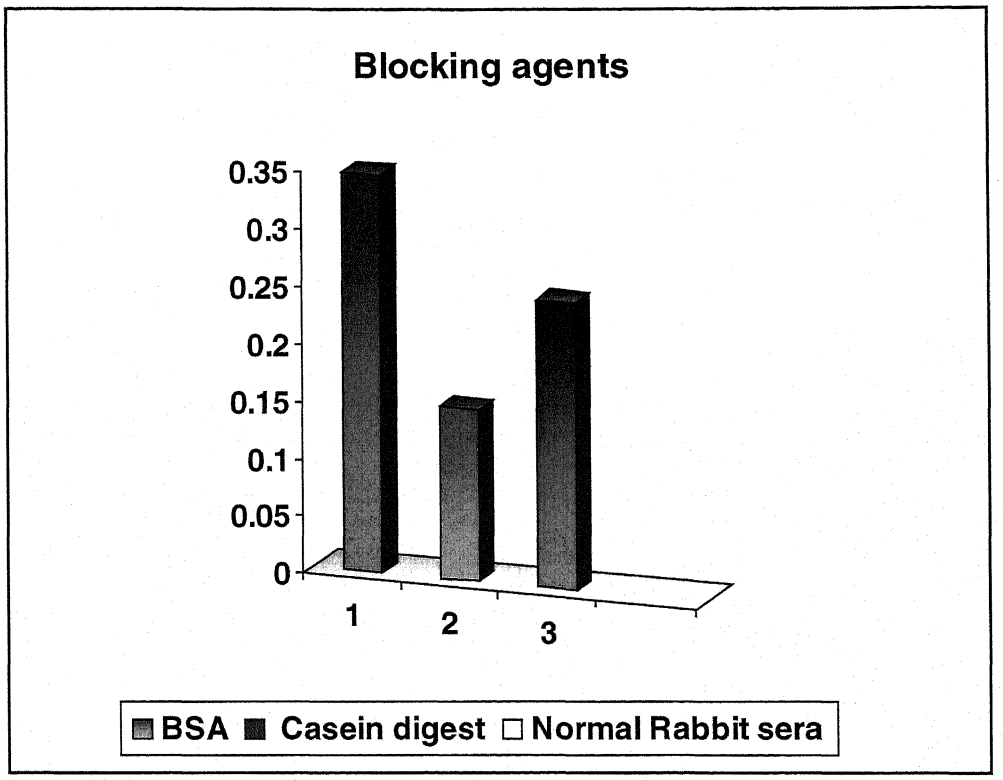

Figure 24: Effect of blocking agents on the specificity of antigen with antibody

Effect of pH of the coating buffer on the immobilization of antigenic secretory protein antigens

All pure secretory protein(s) showed maximum adsorption on solid phase at pH 7.2. Although no significant difference form the adsorption of secretory protein between pH 7.2 and pH 9.5. However, the adsobtion was weak at pH 6.5 as is evident from low absorbance values of all proteins at this pH. Hence, pH7.2 was selected for the immobilization of purified secretory protein and cocktail of antigens (Table – IV and Figure- 25).

Table - IV

Immobilzation antigens (Pure Protein)	Reactivity pH 6.5	Reactivity pH 7.2	Reactivity pH-9.5
64KDa	0.802	1.103	1.102
38KDa	0.791	1.521	1.103
30KDa	0.864	1.810	1.805
26KDa	0.782	1.251	1.103
06KDa	0.879	1.424	1.405
Cocktail of antigen	0.853	2.523	2.164

The 64kDa secretory protein showed maximum adsorption on solid phase at pH 7.2 and pH 9.5. 38kDa secretory protein showed maximum adsorption on solid phase at pH 7.2, 30kDa secretory protein at pH 7.2 and 9.5, 26 kDa secretory protein showed maximum adsorption on solid phase at pH 7.2, and 06kDa secretory protein showed maximum adsorption on solid phase at pH 7.2and 9.5.Cocktail of secretory protein showed maximum adsorption on solid phase at pH 7.2.

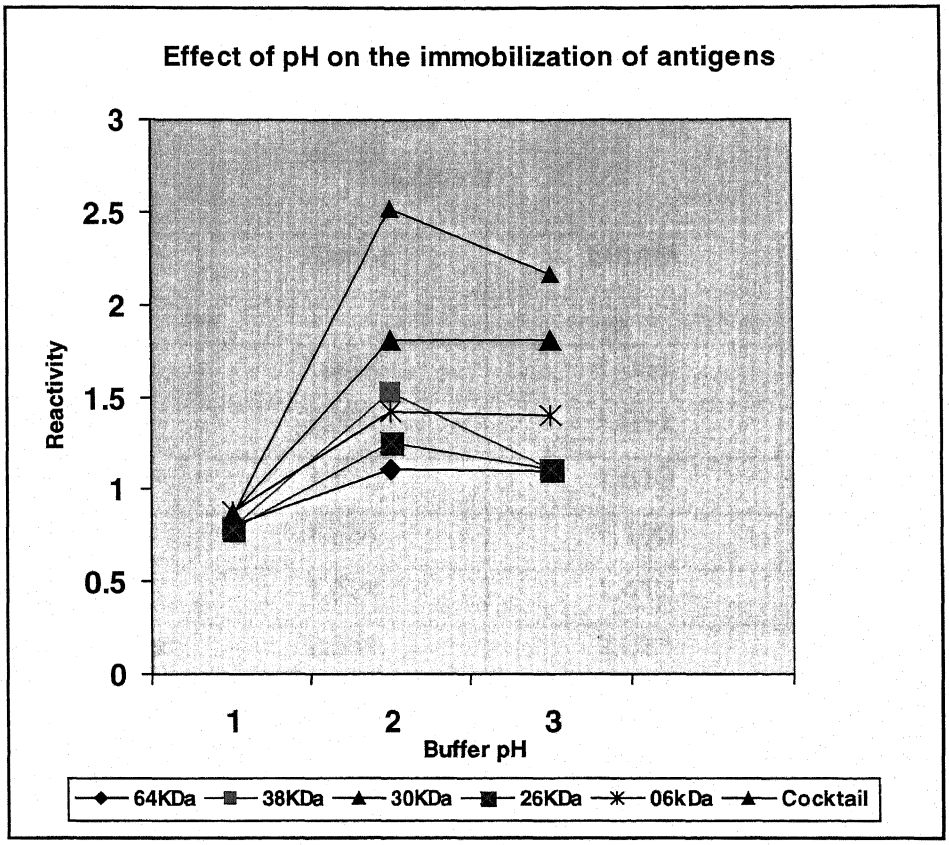

Figure 25: Effect of pH on immobilization of antigenic secretory protein on solid phase. 1: for 64kDa, 38kDa, 30 kDa, 26kDa, 06 kDa, and cocktail at pH 6.5, 7.2 and 9.5.

Effect of ionic strength of Coating Buffer on the immobilization of the selected antigenic Secretory protein antigens

Farther, the ionic strength of coating buffer indicated that all purified proteins and cocktail of antigen bound to the surface and showed good absorbance at all three concentrations (10mM - 100mM). However, it was best at 10mM concentration with a gradual decrease upto 100mM concentration (Table- V and Figure -26).

Table - V

Pure protein antigens	10mM	50mM	100mM
64KDa	1.212	1.051	0.923
38KDa	1.321	1.251	0.910
30KDa	1.933	1.513	0.893
26KDa	1.351	1.100	0.873
06KDa	1.624	1.412	0.926
Cocktail of antigen	2.531	2.021	0.844

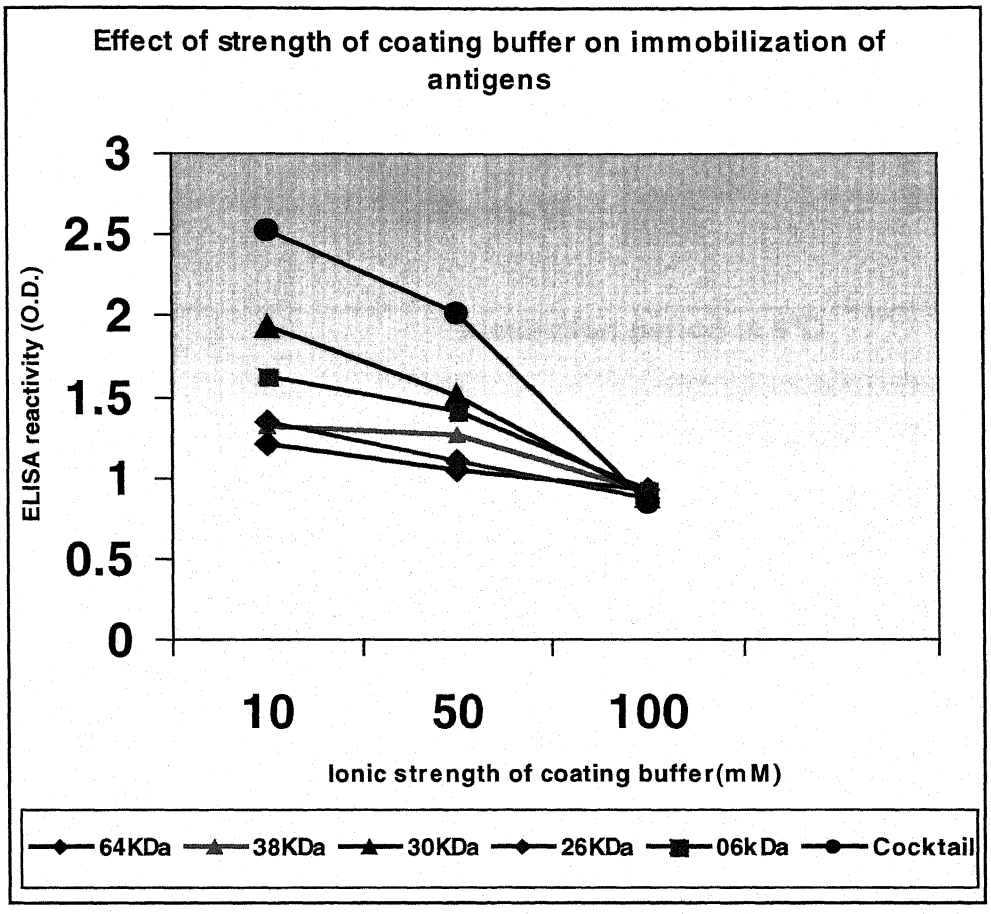

Figure 26: Effect of ionic strength of coating buffer on immobilization of antigenic secretory protein on solid phase.

Effect of incubation period on the immobilization

Effect of incubation period for the antigens to bind on to the solid surface was determined by incubating all purified proteins and cocktail of all these antigens for different time period. As shown in Table-VI and Figure- 27, the absorbance values increased with increasing incubation time and were maximum for the combination, when antigens were kept for immobilization for 18 hours.

Table- VI

Antigens	Incubation period at 4°C			
	3 hour	5 hour	12 hour	18hour
64KDa	0.041	0.133	0.835	1.550
38KDa	0.031	0.212	0.721	1.712
30KDa	0.042	0.222	0.810	1.893
26KDa	0.037	0.116	0.803	1.876
06KDa	0.035	0.102	0.605	1.795
Cocktail of antigen	0.140	0.197	0.799	2.608

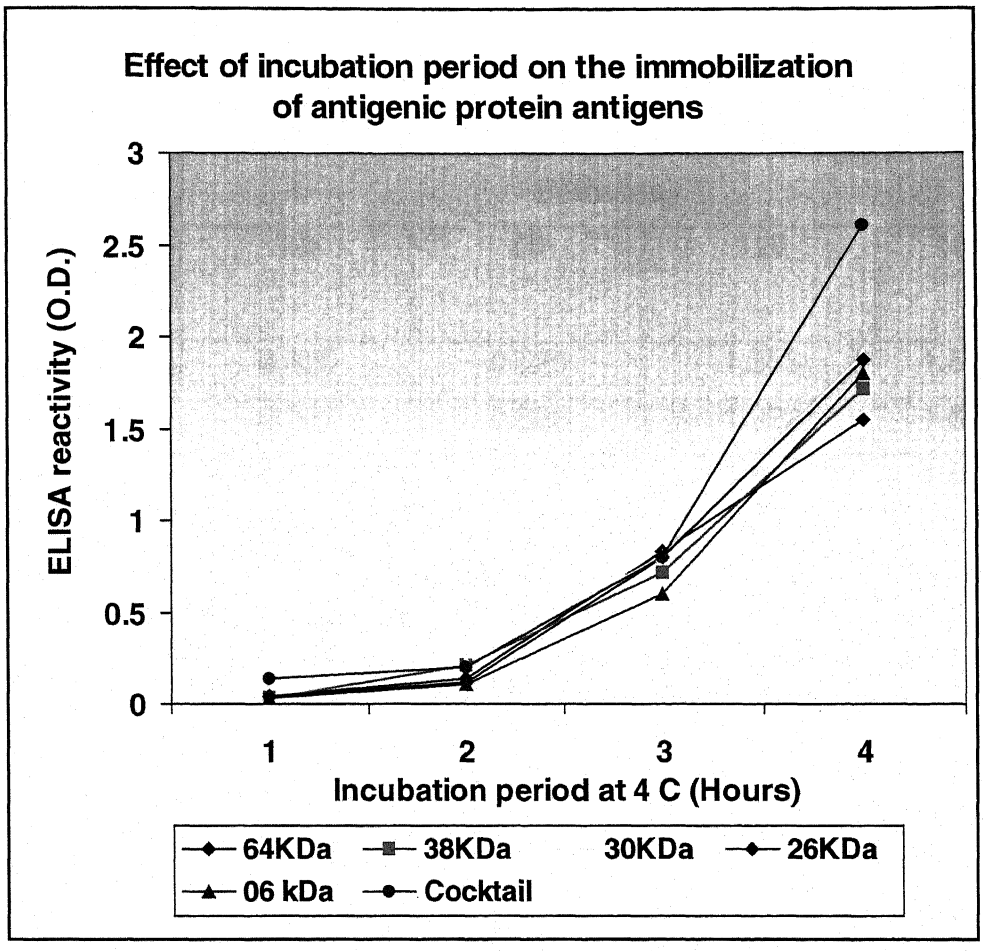

Figure 27: Effect of incubation period on the immobilization of protein antigen and cocktail of antigens.

Further, there was rise in the adsorption of the protein antigen on the solid surface up to 120 minutes at 37 ^{O}C thereafter no further increase in adsorption was observed (Table – VII and Figure- 28) gets saturated.

Table - VII

Antigens	Period of immobilization at 37°C (Minutes)			
	30	60	120	180
64KDa	0.335	0.525	1.345	1.525
38KDa	0.321	0.622	1.322	1.622
30KDa	0.410	0.713	1.813	1.853
26KDa	0.253	0.653	1.650	1.652
06KDa	0.205	0.605	1.615	1.605
Cocktail of antigen	0.599	0.901	2.601	2.701

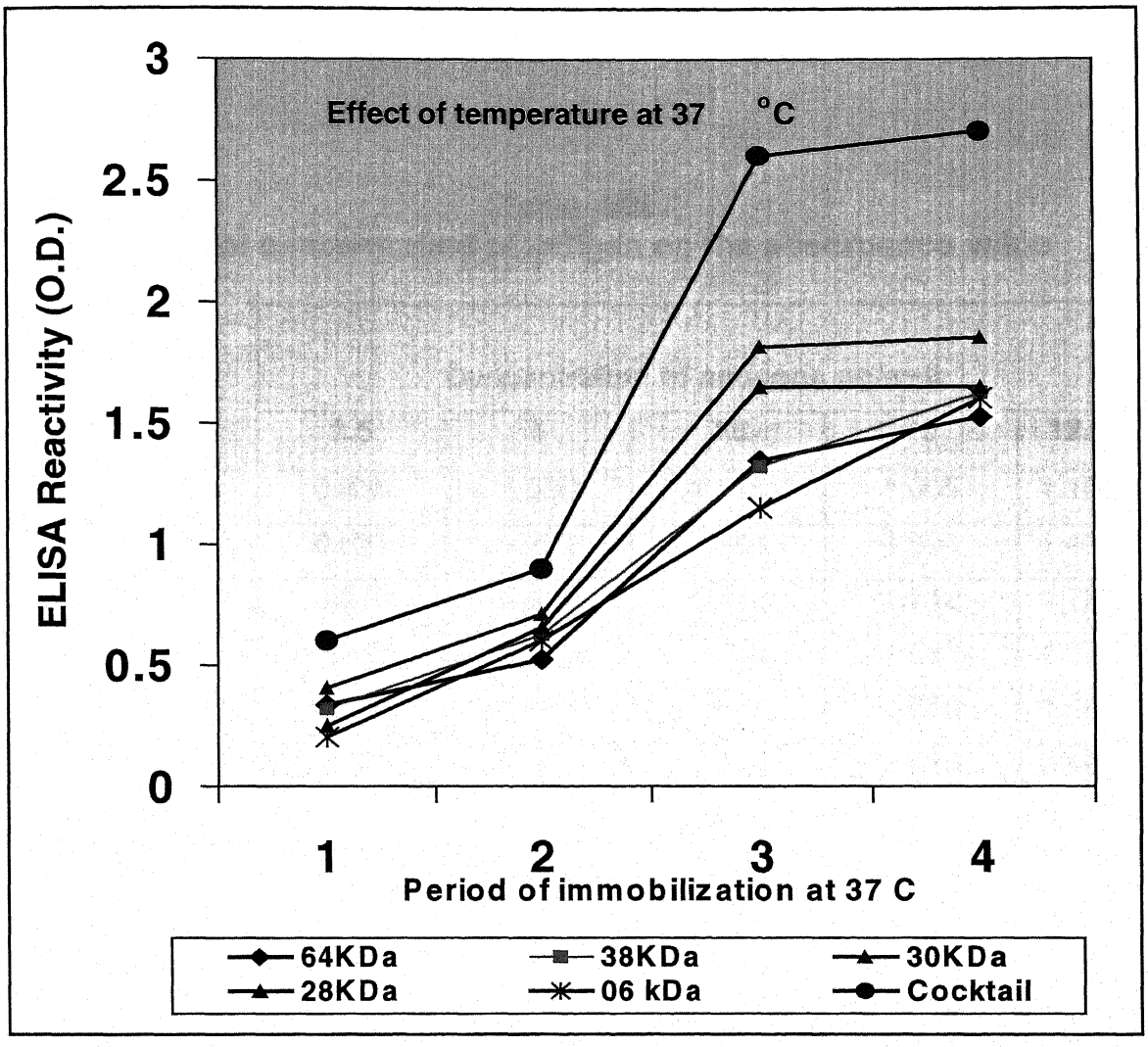

Figure 28: Effect of incubation period of antigens for immobilization at 37°C.

Effect of concentration of antigens

An increase in the concentration of all the protein antigens from 2.5µg / well to 7.5 µg / well demonstrated increase in the absorbance value (Table – VIII and Figure - 29). No further increase in the absorption was observed by increasing the (10µg / well, 12.5µg / well) concentration of antigen for coating.

Table -VIII
Effect of concentration of antigen on the absorbance value

Antigens	Concentration of antigens µg/well				
	2.5	**5**	**7.5**	**10**	**12.5**
64KDa	0.835	1.535	1.515	1.005	1.105
38KDa	0.821	1.721	1.701	1.501	1.402
30KDa	0.910	1.915	1.812	1.712	1.782
26KDa	0.853	1.753	1.743	1.682	1.672
06KDa	0.605	1.505	1.510	1.500	1.507
Cocktail of antigen	0.999	2.599	2.535	2.413	2.403

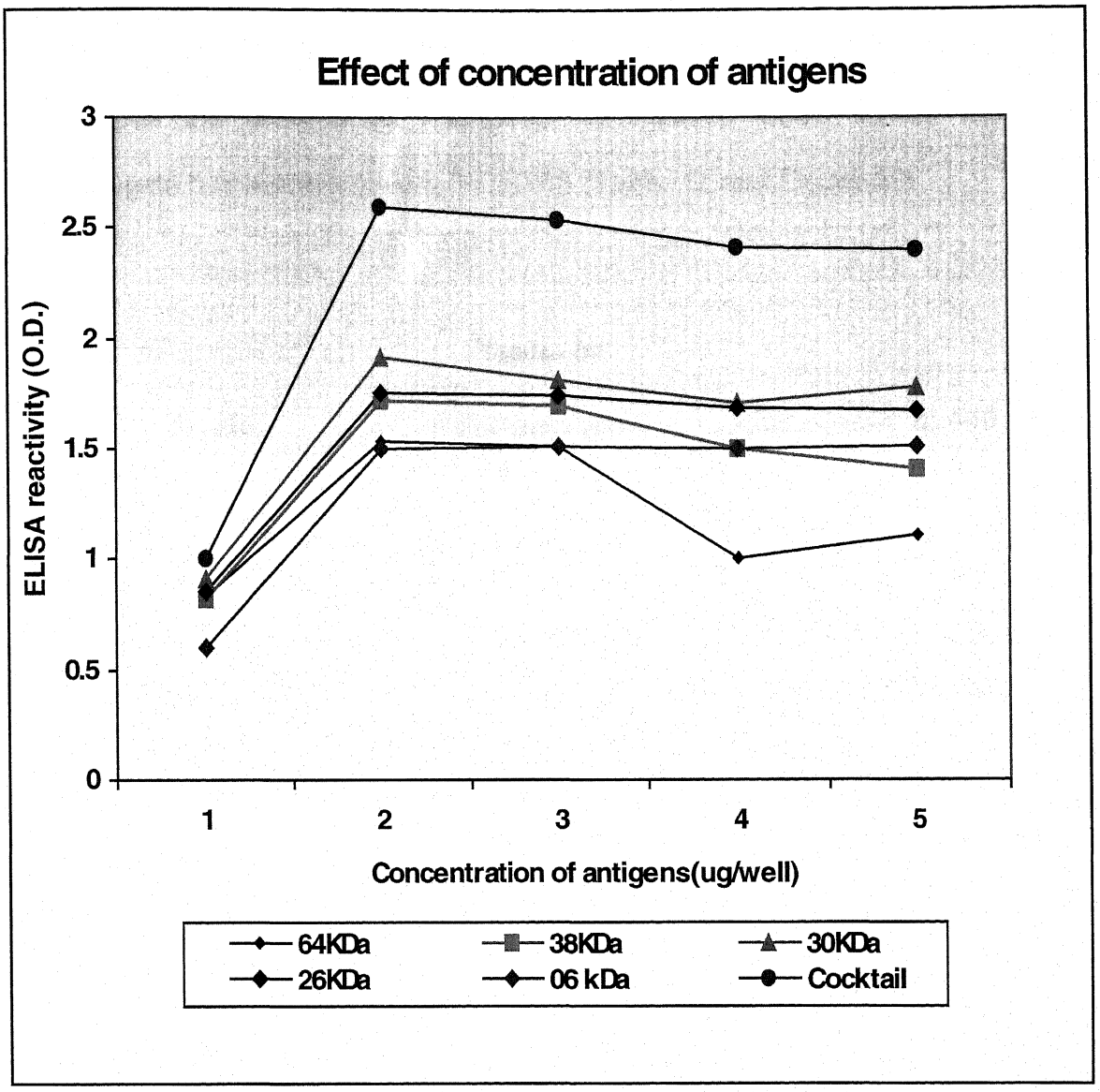

Figure 29: Effect of concentration of antigen on binding to solid surface.

Optimization of conjugate Goat anti-human IgG-HRP

The extent of the reaction exceeds the absorbance 1.0 within 10 minutes and exceeds the absorbance 1.9 within 30 minutes which shows that the conjugate has suitable activity for ELISA (Table- IX and Figure-30).

Table- IX

Sr. No.	Time (minutes)	Absorbance at 450 nm
1	02-03	0
2	05	0.672
3	10	1.123
4	15	1.338
5	20	1.593
6	25	1.793
7	30	1.921

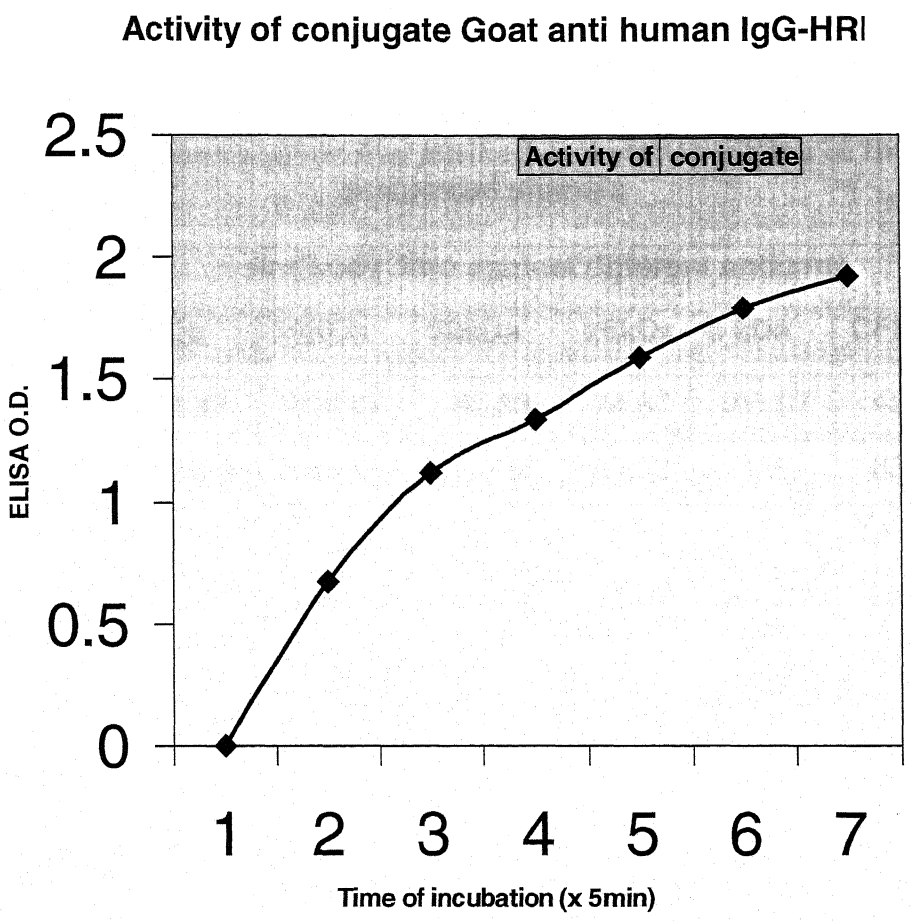

Figure 30: Effect of enzyme reaction time on the extent of the Reaction.

Humoral Response to the Secretory Proteins

Purified proteins were screened for level of antibodies induced against them in the sera of animals immunized with total culture filtrate. It is evident from the Figure- 31 and Table-X that significant level of antibody was found against all the five Secretory proteins as measured by ELISA.

Table - X
Humoral Response against five purified secretory proteins in the sera of immunized animals

Weeks P. im.	Antibody Titre against different antigens					
	64kDa	38KDa	30kDa	26kDa	6 kDa	CFP
1	213.33	298.67	85.33	74.67	106.67	426.67
2	682.67	682.67	106.67	85.33	128	853.33
3	1706.67	2730.67	170.67	64.00	149.33	3413.33
4	2730.67	3413.33	341.33	42.67	106.67	5461.33

The antibody level i. e. $\log_{10}$ antibody titre increased from first week post immunization (p. im.) upto fourth week post immunization against all the antigens. It was significantly higher ($p<0.001$) than control at all time point. The optimum response was obtained at 3rd week p. im. (Table-X) when the antibody titre was maximum. The antibody titre was highest for two proteins i. e. 64 kDa and 26 kDa proteins among all the six protein at all time points.

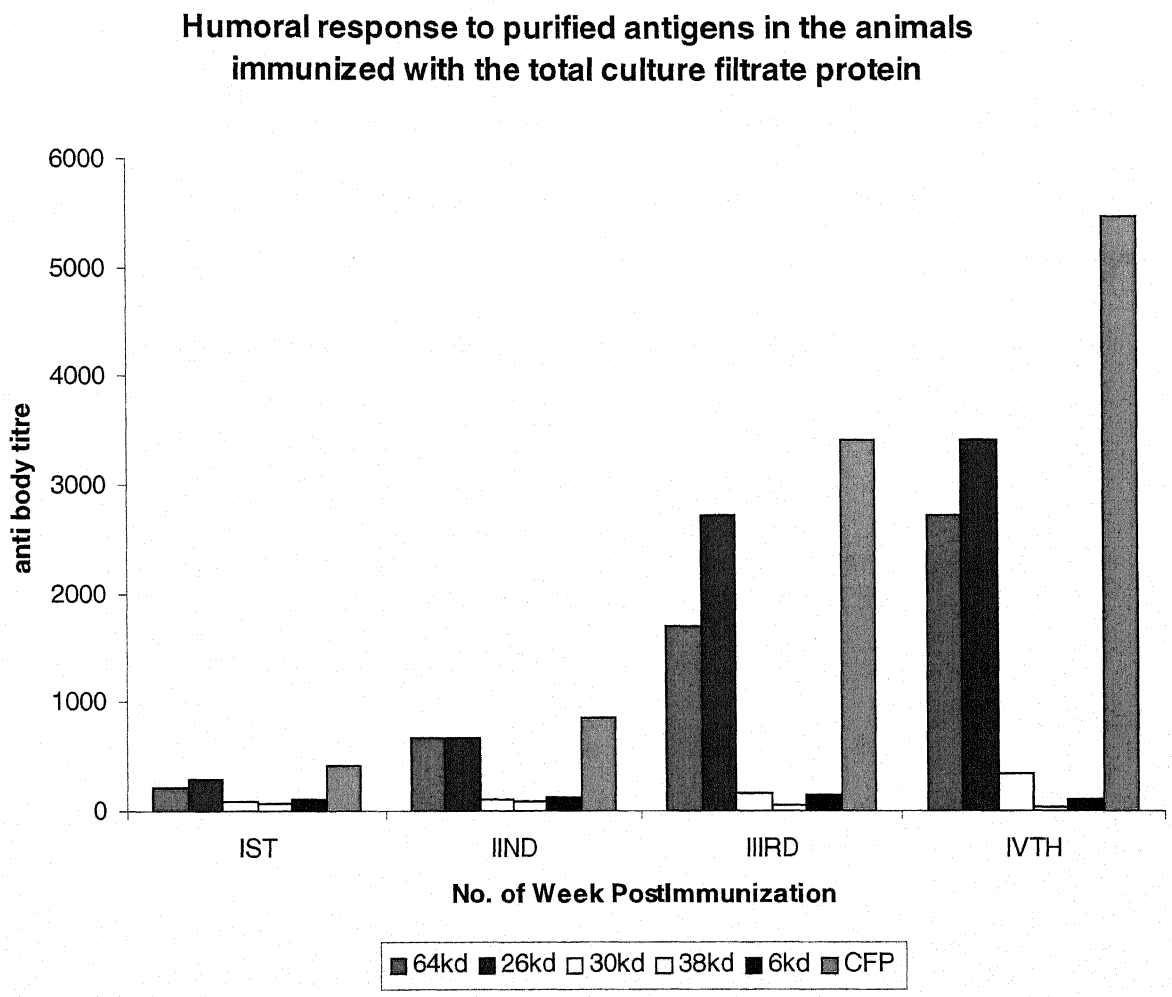

Figure 31: Humoral response to purified antigens in the animals immunized with the total culture filtrate protein

Cell Mediated Immune Response

Cell mediated response to the five proteins was also studied in the lymphocytes obtained from animals immunized with total culture filtrate proteins by T- cell proliferation assay.

T-Lymphocyte response to the pure proteins

The T-lymphocyte proliferation activity was determined in the animals immunized with total culture filtrate protein upto 4-week post immunization for the selection of most immunoreactive protein among the five purified proteins. It is clear from results shown in Figure- 32 and Table -XI that significant T–cell response was observed against all antigens compared to control from 2nd week post immunization (P. im.) up to fourth week post immunization.

Table -XI

Cellular immune Response against five purified secretory proteins in the sera of animals immunized with total culture filtrate.

Weeks	Stimulation Index against different purified antigens					
P. im.	64kDa	38KDa	30kDa	26kDa	6 kDa	CFP
1	1.57±0.40	2.39±0.34	3.4±0.23	1.74±0.39	3.74±0.12	3.33±0.20
2	1.58±0.04	2.87±0.16	4.31±0.31	2.61±0.48	4.95±0.18	3.8±0.37
3	2.59±0.39	3.14±0.06	6.47±0.38	2.68±0.45	7.19±0.35	5.87±0.19
4	1.93±0.44	2.39±0.23	7.02±0.58	1.72±0.27	7.37±0.28	4.64±0.83

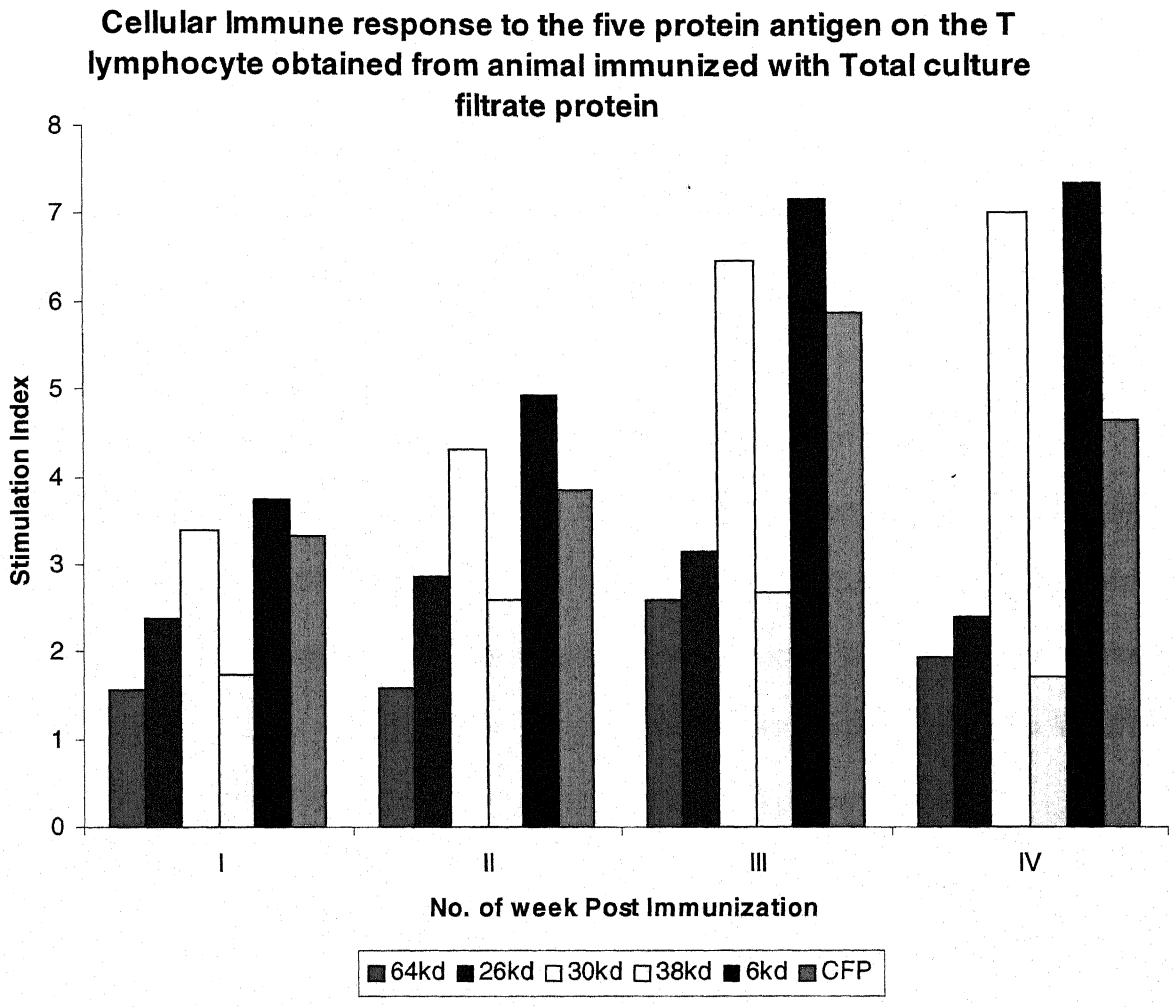

Figure 32: Cellular Immune response to the five protein antigen on the T lymphocyte obtained from animals immunized with Total culture filtrate proteins.

Maximum response of T-lymphocyte proliferation was observed for antigens having Molecular weight of 30 and 6 kDa respectively and was highest for 6kDa protein.

On the basis of humoral response and cell mediated response of the purified culture filtrate proteins, two antigens viz. 64 and 26 elicited maximum antibody induction and antigens 30 and 6 kDa elicited maximum T–lymphocyte proliferation. These proteins viz. 64 and 26 and 6 kDa respectively were, therefore, selected for further B and T-lymphocyte studies.

Immunogenicity of 6kDa Secretory protein complexed in FIA

The ability of 6kDa protein to induce response was determined by immunizing mice with three different concentrations (75, 37.5, 18.75 µg / mouse) in three doses. After completion of immunization T-lymphocyte proliferation response to the immunized cell with 6kDa antigen was studied to determine the level of T–cell activity indicating the immunogenic potential of the protein.

Cell Mediated Immune Response

As explained earlier, cell mediated response was determined by T–lymphocyte proliferation assay. Animals immunized with 6kDa antigen complexed in IFA exhibited a dose dependent proliferative response during the study period. The proliferation level was low ($p < 0.05$) in first week post immunization (Figure – 33 and Table -XII), which increased thereafter, and reached its peak level after 3 weeks (6.26±0.16, 9.04±0.54, 12.56±1.07) P. im compared to control and remained constant till fifth week.

Table - XII

T-Lymphocyte Proliferative Response to 6kDa Secretory Protein in Animal Immunized with Different Concentration of 6kDa-IFA

Weeks P. im.	Stimulation Index against different concentration of 6kDa antigens		
	18.75 µg	37.5 µg	75 µg
1	2.96±0.08	3.03±0.16	3.59±0.35
2	4.74±0.37	6.03±0.39	6.39±0.43
3	5.63±0.45	7.38±0.28	9.36±0.37
4	6.26±0.16	9.04±0.54	12.56±1.07

Values represented are Mean ± SD of 3-5 animals

Comparison of Immune Response Elicited to 6kDa Secretory Protein in 6kDa IFA Immunized Animals and BCG Vaccinated Animals.

The animal immunized with 6 kDa-IFA elicited maximum cellular response at 75µg concentration after 3 weeks post immunization. The immune response induced in mice immunized with 6kDa-IFA was compared to those immunized with live BCG vaccine. Cellular response to 6kDa secretory protein in immunized (6 kDa-IFA), and BCG vaccinated and control animals were studies upto fourth week p. im.

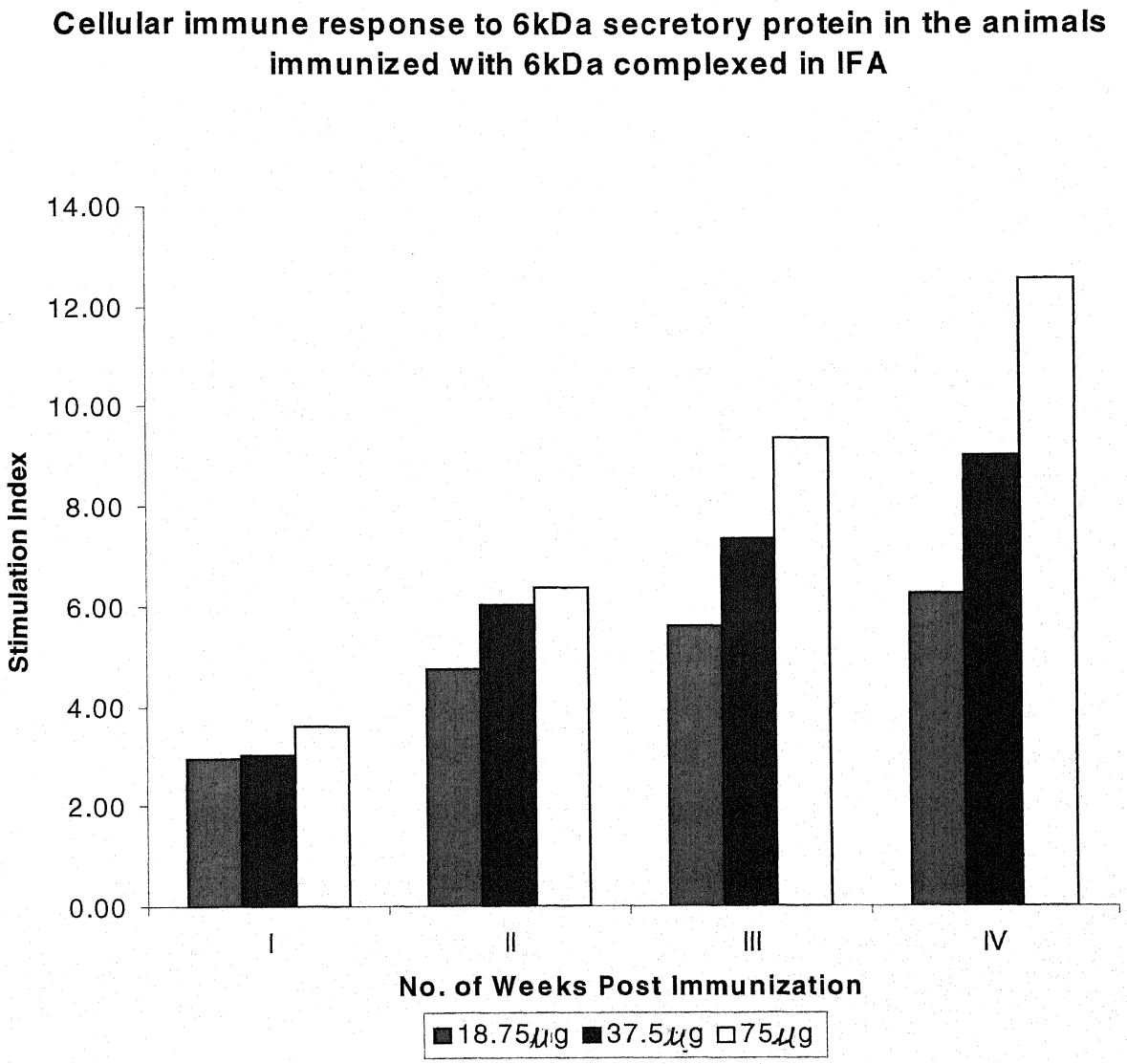

Figure 33: Cellular immune response to 6kDa secretory protein in the animals immunized with 6kDa complexed in IFA

Cell Mediated Immune Response

The induction of T-lymphocyte activation to 6kDa antigen in both the groups (6kDa-IFA and BCG vaccinated) was monitored by T-lymphocyte proliferation assay.

T-lymphocyte Proliferation Assay

The T-lymphocyte proliferation induced in 6kDa-IFA immunized and BCG vaccination mice is represented in Figure - 34 and Table -XIII.

Table: XIII

T- Lymphocyte Proliferation Response in Animals Immunized with 6kDa-IFA/ BCG Vaccinated

Weeks P. im.		Stimulation Index against 6kDa antigen	
	75 µg	BCG	Control
1	3.67±0.31	2.96±0.08	1.44±0.24
2	6.45±0.33	4.62±0.09	1.24±0.14
3	9.60±0.41	5.25±0.09	1.23±0.24
4	13.07±0.78	5.82±0.20	1.52±0.31

Values represented are Mean ± SD of 3-5 animals in each group

The T-lymphocyte response increased from 1st week p. im. (3.67±0.31, 6.45±0.33) upto 3rd p. im. (9.60±0.41) and reached its peak in the fourth week p. im. (13.07±0.78) in 6kDa-IFA and BCG vaccinated animals but remained significant as compared to control animals ($p<0.01$). However, the animals immunized with 6kDa-IFA exhibited significantly higher T-lymphocyte as compared to BCG vaccinated animals, thus indicating that the 6kDa purified antigen has ability to induce strong T-cell proliferation, which is a pre requisite for protective immune response.

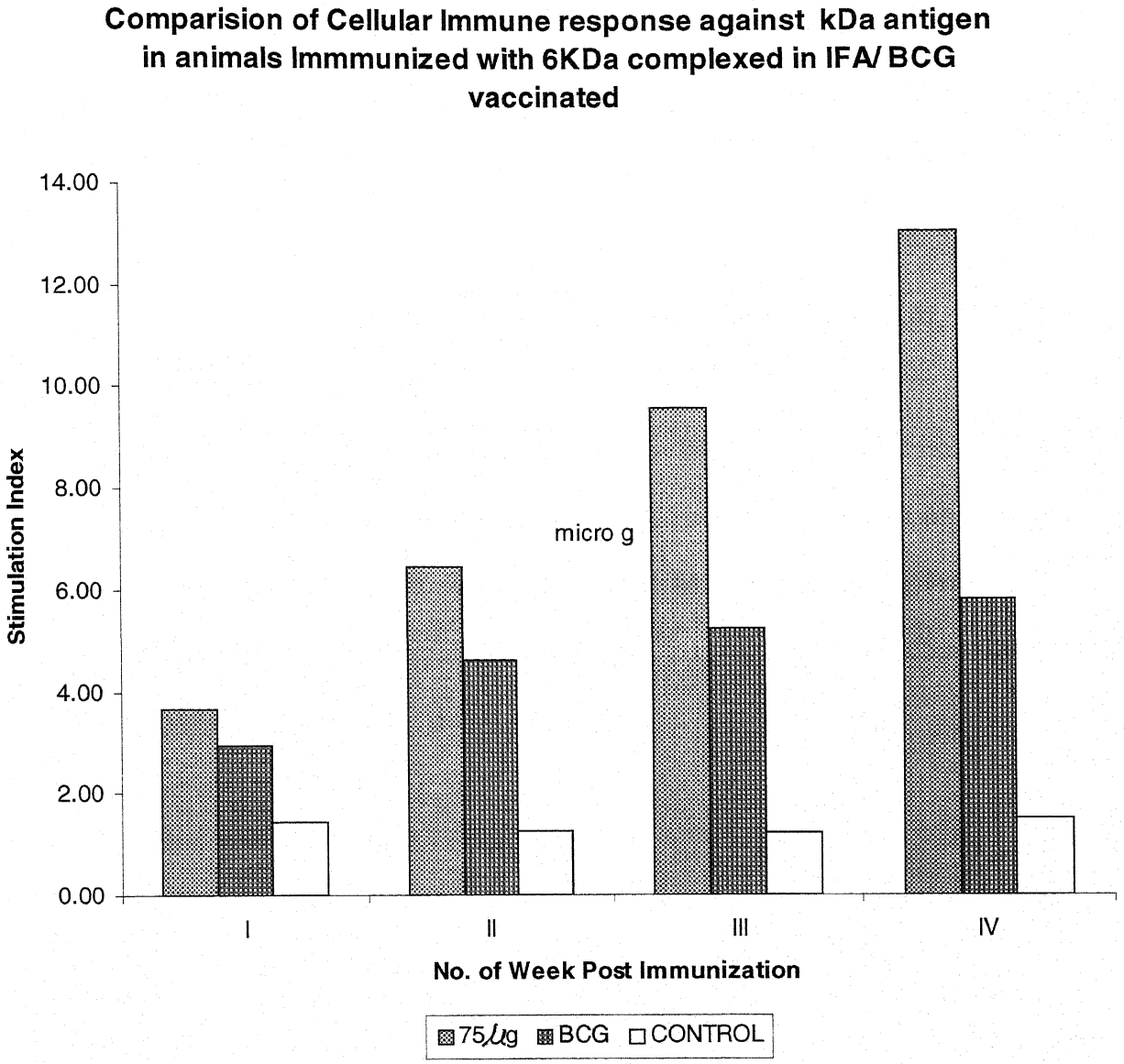

Figure 34: Comparision of Cellular Immune response against kDa antigen in animals Immmunized with 6KDa complexed in IFA / BCG vaccinated

Immunoprotective activity of 6kDa purified protein

The ability of 6kDa protein to induce strong CMI response encouraged us to determine the role of this antigen in protecting the immunized mice against lethal challenge of *M. tuberculosis.*

Determination of Lethal Dose 50 (LD_{50}) for animals infected with *M. tuberculosis* $H_{37}Rv$

The mice were infected intravenously with different concentration of *M. tuberculosis* $H_{37}Rv$ to determine the LD_{50} . A total of 10 mice in each group were injected with different concentrations of *M. tuberculosis* $H_{37}Rv$ and the mortality in each group was recorded upto 30 days post infection for the calculation of LD_{50} (Table- XIV).

Table: XIV

LD_{50} for Mice Infected Intravenously with *M. tuberculosis* $H_{37}Rv$

No. of CFU's /mouse	No of mice Infected	Mortality after 30 days	Percent	Calculation
3.6×10^8	11	11	100	**LD50= 2.93 x 107 CFU/mouse**
3.6×10^7	10	6	60	
3.6×10^6	10	2	20	
3.6×10^5	8	1	12.5	
3.6×10^4	8	0	0	

The LD_{50} was found to be 3×10^7 colony forming units per mouse and this dose was used throughout the study for induction of experimental tuberculosis.

Protection Afforded by Immunization with 6kDa-IFA and comparison with BCG vaccine

Immunization with 6kDa secretory protein elicited strong cellular response, which was maximum in the 3rd week post immunization. Hence, to study the protective efficacy the mice were immunized with different concentration (75µg, 37.5 µg and 18.75 µg per mouse) of 6kDa secretory protein complexed in IFA and live BCG vaccine $H_{37}Rv$ (1×10^6 CFU's per mouse). The immunized and unimmunized control mice were challenged after three weeks post immunization (p. im.) with LD_{50} (3.0×10^7 CFU's / mouse) of *M. tuberculosis* $H_{37}Rv$. The protection imparted by the 6kDa protein and BCG vaccine was estimated by monitoring the survival rate in immunized / control mice upto 30 days post challenge.

Percent Survival

The relative percent survival of immunized / control mice challenge with LD_{50} of *M. tuberculosis* has been depicted in Figure- 35 and Table –XV.

Table- XV

Protective effect (On Survival basis) in the Animals Immunized with 6kDa-IFA / BCG vaccine and Challenged for four weeks p. im. with *M. tuberculosis* $H_{37}Rv$

Groups Immunized with	Protective effect= No. of survival/No. tested on various Post Challenged days			
	7 day	14 day	21 day	30 day
6kDa (75 µg)	20 / 20(100)*	20 /20(100) *	16 / 20(80) *	15 / 20(75) *
6kDa (37.5 µg)	20 / 20(100) *	20 /20(100) *	14 / 20(70) *	10 / 20(50) *
6kDa (18.75 µg)	20 / 20(100) *	20 /20(100) *	12 / 20(60) *	8 / 20 (40) *
BCG vaccine	20 / 20(100) *	20 /20(100) *	16 / 20(80) *	14 / 20(70) *
Control	20 / 20(100) *	20 /20(100) *	10 / 20(50) *	6 / 20 (30) *

* Figure in parentheses are percent survivors at different time points

The control animals (immunized with normal saline-FIA) exhibited a steady decline in percent survival and at thirty days post challenge only 30% of the control mice survived the challenge. On the other hand, the mice immunized with varying concentration of the antigen demonstrated no mortality till two weeks post challenge. However, the onset of mortality occurred during third week post challenge with 20%, 30%and 40% mortality in animals immunized with 75µg, 37.5 µg, 18.75µg of the antigen respectively and 20 % mortality was also observed in animals vaccinated with BCG vaccine. In the fourth week an increased mortality (50% to 60%) was observed in the lower two doses of 6kDa immunized animals. Mortality was also observed in 75µg protein (25%) and BCG vaccinated (30%) animals.

At the end of 30 days observation, 75% survival was seen in animals immunized with 75µg concentration of 6kDa protein that was similar to survival (70%) afforded by BCG vaccine.

Thus, the result indicates that 6kDa protein possess immunoprotective behavior and protection induced by the highest dose of the antigen greater than BCG vaccine.

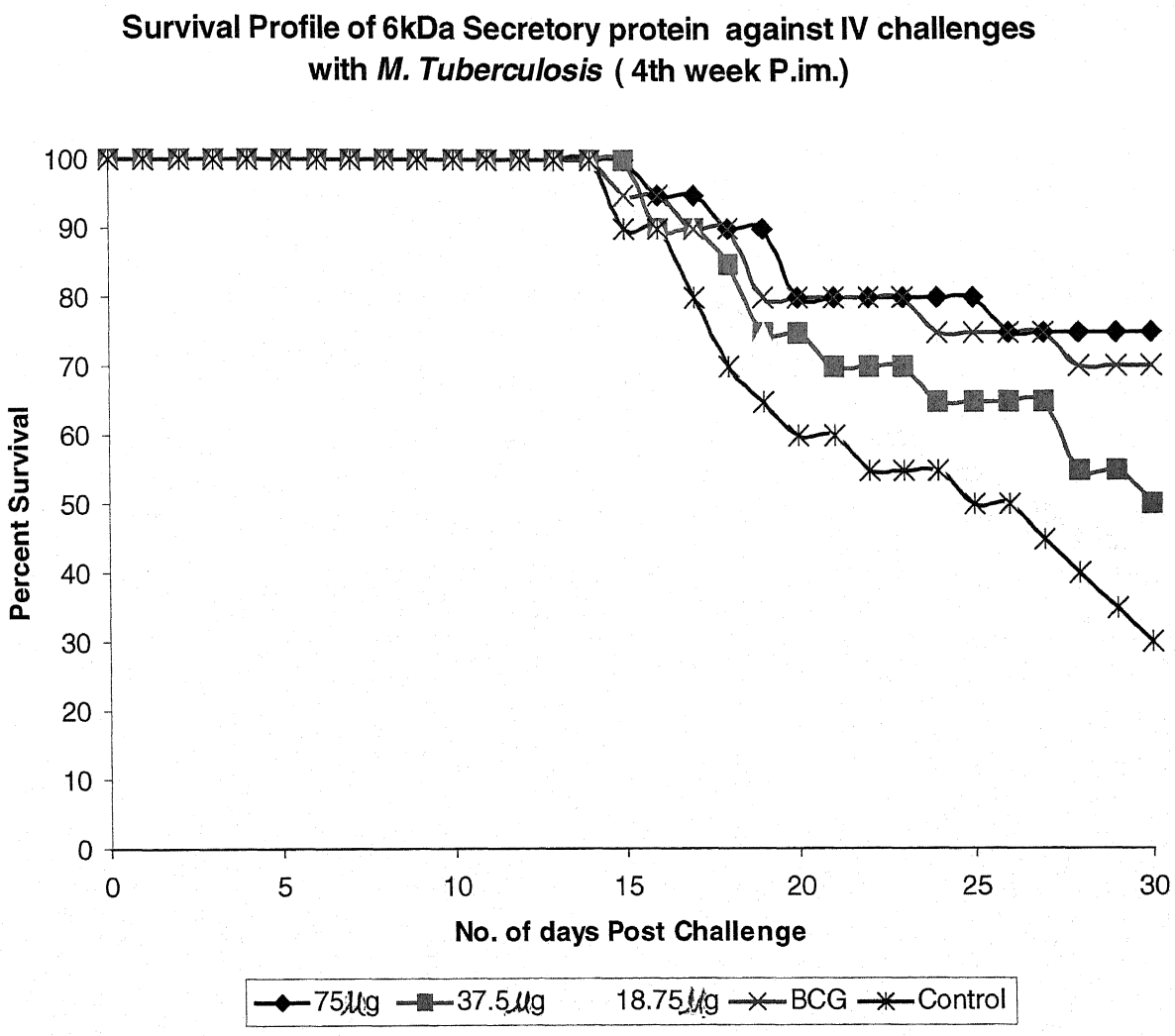

Figure 35: Survival Profile of 6kDa Secretory protein against IV challenges with *M. tuberculosis* (4^{th} week P. im.).

Enumeration of Viable bacilli

The protection imparted by 6kDa secretory protein and BCG vaccine was also assessed by determining the clearance of *M. tuberculosis* $H_{37}Rv$ from the organs (Liver, spleen and lung) and its comparison with unimmunized animals.

Spleen

At the end of observation period i. e. 30 day post challenge, there was approximately 200-300 fold decrease in the viable counts in spleen (Table-XVI) of both 6kDa-IFA immunized ($2.1\pm 0.11\times10^6$) and BCG vaccinated ($2.45\pm 0.71\times10^6$) mice as compared to control ($5.8 \pm 0.18\times10^8$) which was highly significant ($p<0.001$).

Table- XVI

Viable *tubercle bacilli recovered* from 6kDa Immunized / BCG Vaccinated and Control Animals Challenged 4weeks p. im. with LD_{50} of *M. tuberculosis* $H_{37}Rv$

Groups immunized with	CFU recovered from target organs		
	Lung	spleen	Liver
6kDa IFA (75 µg)	$2.5 \pm 0.12\times10^{6***}$	$2.1\pm 0.11\times10^{6***}$	$4.7\pm 0.16\times10^{6***}$
BCGVaccinated	$3.9 \pm 0.17\times10^{7**}$	$2.45\pm 0.71\times10^{6***}$	$3.07\pm 0.89\times10^{6***}$
Control	$3.4 \pm 0.11\times10^8$	$5.8 \pm 0.18\times10^8$	$6.5 \pm 0.10\times10^8$

***p<0.001 level, **p<0.01 level

Lung

The number of organisms present after 30days post challenge in the lungs of Control, 6kDa-FIA immunized and BCG vaccinated mice where 3.4± 0.11×10^8, 2.5± 0.12×10^6, 3.9 ± 0.17×10^7 respectively as shown in Table- XVI. There was approximately 100-200 fold decrease ($p<0.001$) in CFU's in 6kDa-IFA immunized and 10 fold ($p<0.01$) in BCG vaccinated animals as compared to control.

Liver

The CFU's detected in liver of Control, 6kDa-IFA immunized and BCG vaccinated mice after 30 days post challenge are presented in Table 17.There was approximately 100-200 fold decrease in the viable counts in 6kDa-IFA immunized (4.7± 0.16×10^6) and BCG vaccinated (3.07± 0.89×10^6) mice as compared to the control (6.5 ± 0.10×10^8) and the observed differences were highly significant ($p<0.001$).

These results suggest that the protection afforded by 6kDa secretory protein against experimental tuberculosis (Figure - 35 and Table - XV, XVI) was better to that imparted by BCG vaccine both in terms of survival and clearance of bacilli from the target organs at the peak of immune response.

Immune Response in 6 kDa-Lip (Liposome) Immunized Animals

Humoral Immune Response

Results obtained (ELISA antibody titre) with immunized/ control sera at various week post immunization have been presented in Figure -36, Table XVII.

Table- XVII

Humoral Immune Response in 6kDa-Lip (Liposome) Immunized Animals

Week p. im.	Antibodies titre of 6kDa-Lip in animal immunization	
	6 kDa-Lip	N. Saline-Lip (Control)
1	97.67± 0.47	5.8± 0.47
2	101±0.7	5.8±0.47
3	120.33±0.33	6.51± 0.31
4	101.67± 0.47	6.3±0.5

*Values represented are Mean± SD of 3-5 animals

Significant level of antibodies of 6kDa Secretory protein were detected in sera of immunized mice. The antibodies of 6kDa protein increased from Ist week p. im. (97.67± 0.47) and reached peak level in third week p. im. (120.33±0.33) with slight decline in the fourth week p. im. (101.67± 0.47).

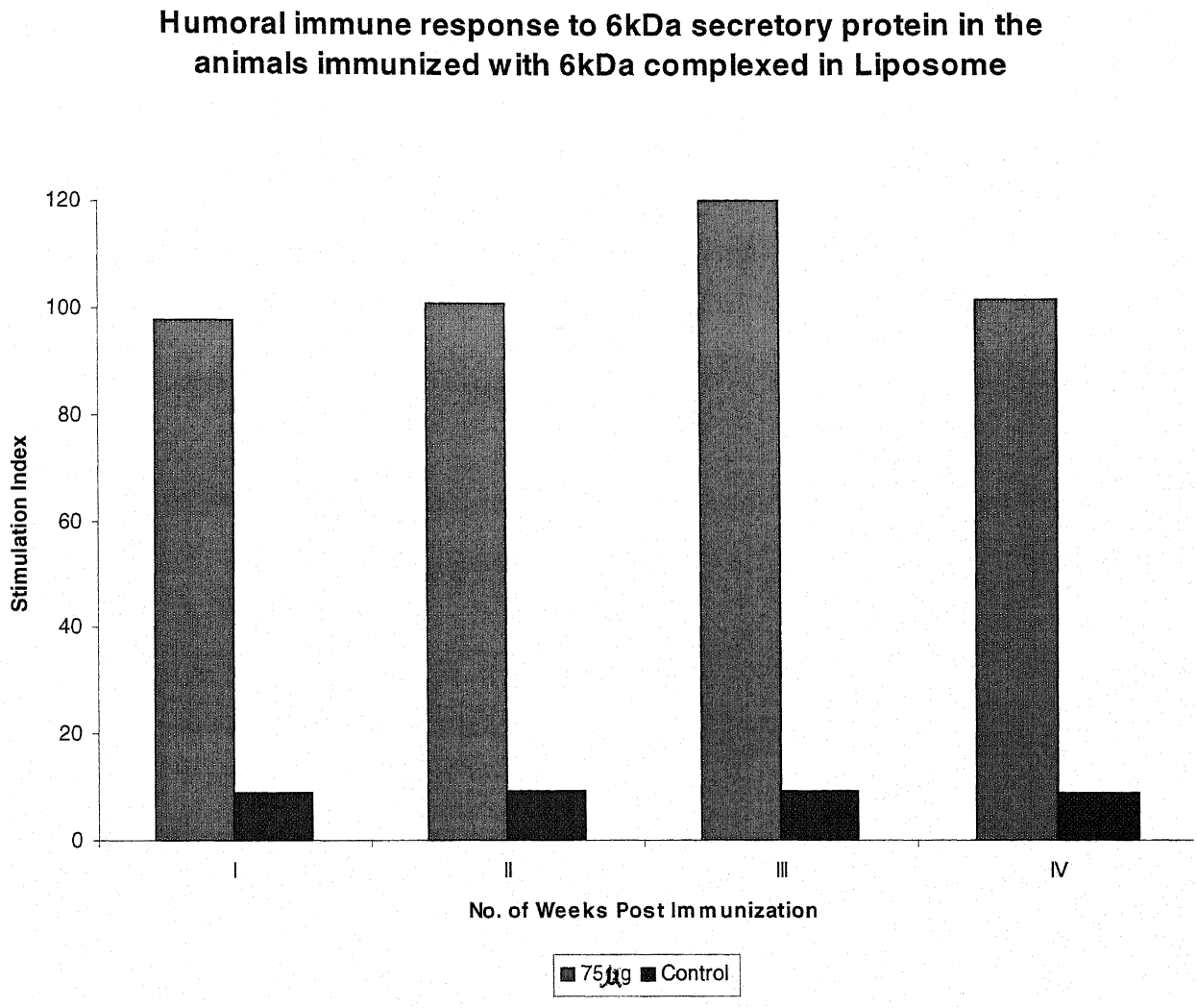

Figure 36: Humoral immune response to 6kDa secretory protein in the animals immunized with 6kDa complexed in Liposome

Cell Mediated Immune Response

The induction of T lymphocyte activated by 6 kDa antigen in 6kDa liposome (kDa-Lip) immunized animals was determined by T-lymphocyte proliferation.

T-lymphocyte Proliferation Assay

The T-lymphocyte proliferation induced in 6 kDa-Lip immunized animals was monitored at various post immunization (p. im.) time intervals and is expressed as stimulation index (Figure - 37 and Table - XVIII).

Table- XVIII

Lymphocyte Proliferative Response to 6 kDa antigen in Animal Immunized with 6 kDa-Liposomes

Week p. im.	Stimulation Index in animal immunized*	
	6 kDa-Lip	N. Saline-Lip (Control)
1	3.10±0.12	1.77± 5.433
2	4.17±0.18	2.74±0.231
3	6.19±0.35	1.42±0.15
4	6.36±0.28	1.60±0.043

The T- cell activity increased from I^{st} week p. im. to third week p. im. (3.10±0.12 to 6.19±0.35) and thereafter declined slightly in 4^{th} week p. im (6.36±0.28). The T-lymphocyte activity in immunized mice was significantly higher ($p<0.001$) than the control animal (1.77± 5.433 to 2.74±0.231) at all the observation time periods.

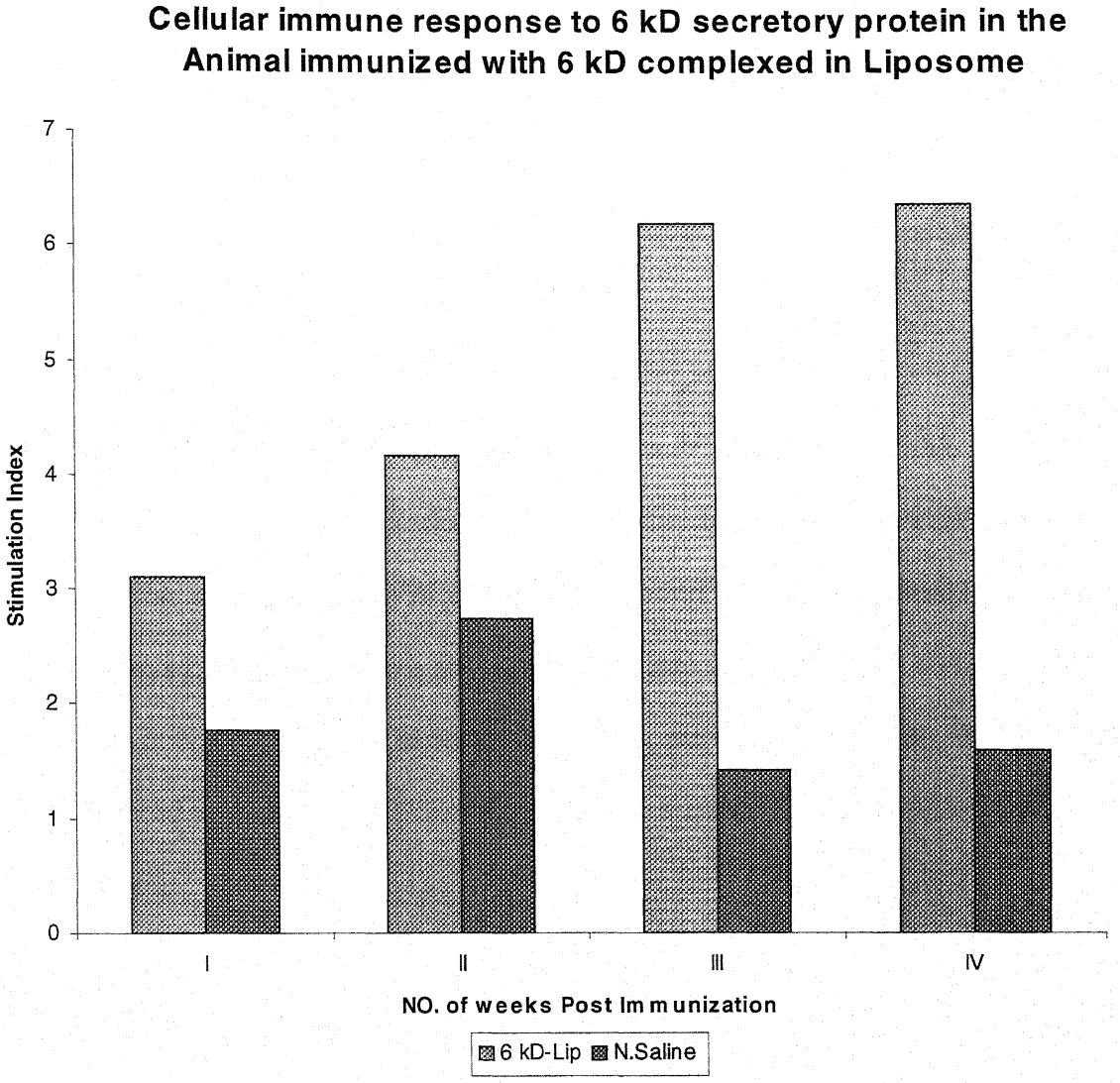

Figure 37: Cellular immune response to 6 kDa secretory protein in the Animal immunized with 6 kDa complexed in Liposome

Protection Afforded by Immunization with 6 kDa Secretory Protein Incorporated in Liposomes (6 kDa-Lip).

Mice immunized with 6 kDa-Lip and unimmunized controls were challenged with LD_{50} (3x 10^7 CFU's) of *M. tuberculosis* $H_{37}Rv$ 4th week after the last immunizing dose. The protection imparted by immunization with 6 kDa-Lip was determined by monitoring the survival rates and viable bacilli enumeration in lungs, spleen and liver.

Percent Survival

The relative in immunized/control group has been depicted in Table - XIX and Figure - 38. The control group exhibited a steady decline in percent survival and at the end of 30 days of observation period only 32% animals survived. The immunized mice did not show mortality upto 2nd week post challenge and at the end of 30 days observation, 76% survival was seen in animals immunized with 6kDa-Lip that was similar to survival (72%) afforded by BCG vaccine.

Table- XIX

Protective effect (On Survival basis) in the Animals Immunized with 6kDa-Lip/ BCG vaccine and Challenged 4th week p. im. with *M. tuberculosis* $H_{37}Rv$

Group Immunized with	Protective effect= No. of survival/No. tested on various Post Challenged days			
	7	14	21	30
6 kDa-Lip(75µg)	25/25(100) *	25/25(100) *	20/25(80) *	19/25(76) *
BCG Vaccinated	25/25(100) *	25/25(100) *	20/25(80) *	18/25(72) *
Control (N. Saline-Lip)	25/25 (100) *	25/25(100) *	12/25(48) *	8/25(32) *

* Figure in parentheses are percent survivors at different time points

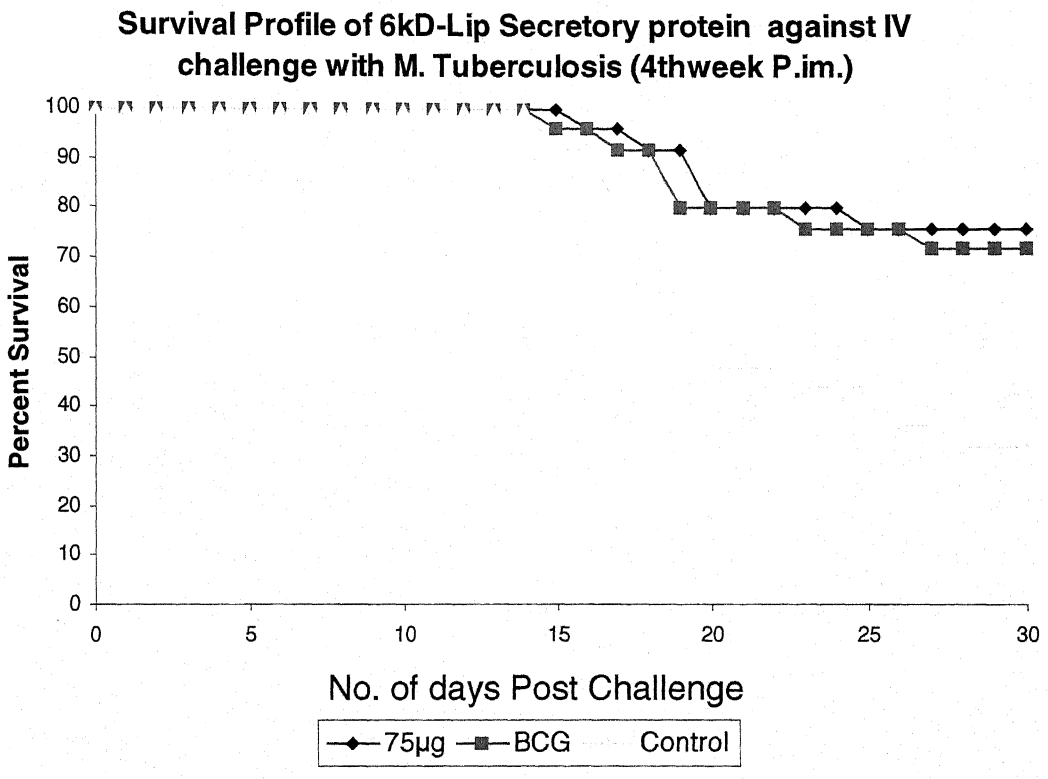

Figure 38: Survival Profile of 6kDa-Lip Secretory protein against IV challenges with *M. tuberculosis*.

Enumeration of Viable bacilli

Number of CFU's present in spleen, liver and lung of control and 6 kDa-Lip immunized mice has been represented in Table- XX.

Spleen

At the time of the observation period i. e. 30 days of challenge, approximately 100 fold decrease in the viable counts was observed in 6 kDa-Lip immunized mice (1.99 ±0.15 $x10^6$) as compared to control (5.2 ±1.2$x10^7$) and the difference observed was highly significant ($p<0.001$).

Table- XX

Viable counts of *M. tuberculosis* $H_{37}Rv$ in Organs of Animals Immunized with 6 kDa-Lip and BCG Vaccine and Challenged 4th week p. im. with *M. tuberculosis* $H_{37}Rv$

Group Immunized with	CFU's (Mean ± SD)		
	Lung	Spleen	Liver
6 kDa-Lip	2.6 ±1.01$x10^{6**}$	1.99 ±0.15 $x10^{6**}$	3.2 ±1.6 $x10^{6***}$
BCG Vaccinated	3.3 ±036$x10^6$	2.60 ±0.28$x10^6$	3.3 ±0.3x 10^7
Control	7.2 ±1.2$x10^7$	5.2 ±1.2$x10^7$	7.3 ±0.98$x10^7$

** $p<0.01$ level

*Values represented are Mean± SD of 3-5 animals

Liver

The number of organisms present in the liver of 6 kDa-Lip immunized and control mice were 3.2 ±1.6 $x10^6$ and 7.3 ±0.98$x10^7$.There was appromixately 200 fold decrease in CFU's in immunized as compared to control mice ($p<0.001$), which was highly significant.

Lung

The CFU's detected in the lungs of infected control and 6 kDa-Lip immunized mice at 30^{th} day post challenge are represented in Table- XX. Approximately 50 fold decreases in viable bacilli was observed in the 6 kDa-Lip immunized mice and this difference was highly significant ($p < 0.001$) compared to controls.

Serodiagnostic potential of cocktail of purified antigens

Since the antibodies to the five purified antigens Viz. 6,26,30,38 and 64 kDa protein were seen in the sera of patients and was also detected in the humoral response in animals, It was worthwhile to test the diagnostic potential of these antigens.

In the present study a cocktail of all five proteins was used to enhance the sensitivity of the assay and attempts were also made to check the specificity of the cocktail.

A total of 427 sera samples were included in this study (Table – XXIand Figure-39) from healthy individuals without any clinical symptoms of TB; these were included as negative controls to evaluate specificity criterion of the test. Most of these sera were obtained from BCG vaccinated normal healthy subjects. The non-TB sera generally belonged either to healthy individuals or to patients suffering from a variety of diseases other than tuberculosis. The sera were stored frozen and were used within one year from the time of their collection.

Table- XXI

Source and status of individuals used in the study

S. No.	Specimens	Numbers of serum sample
1	Pulmonary tuberculosis, serum sample	148
2	Extra-pulmonary, serum sample	50
3	Meningitis tuberculosis, serum sample	14
4	Leprosy	25
5	Malaria, serum sample	18
6	Hepatitis B/C serum sample	11
7	Lung cancer, serum sample	32
8	Bacterial pneumonia, serum sample	14
9	Normal healthy	30
10	BCG Vaccinated, serum sample	58
11	BCG Unvaccinated, serum sample (Normal sample)	27

Total No. of sample collected: 427

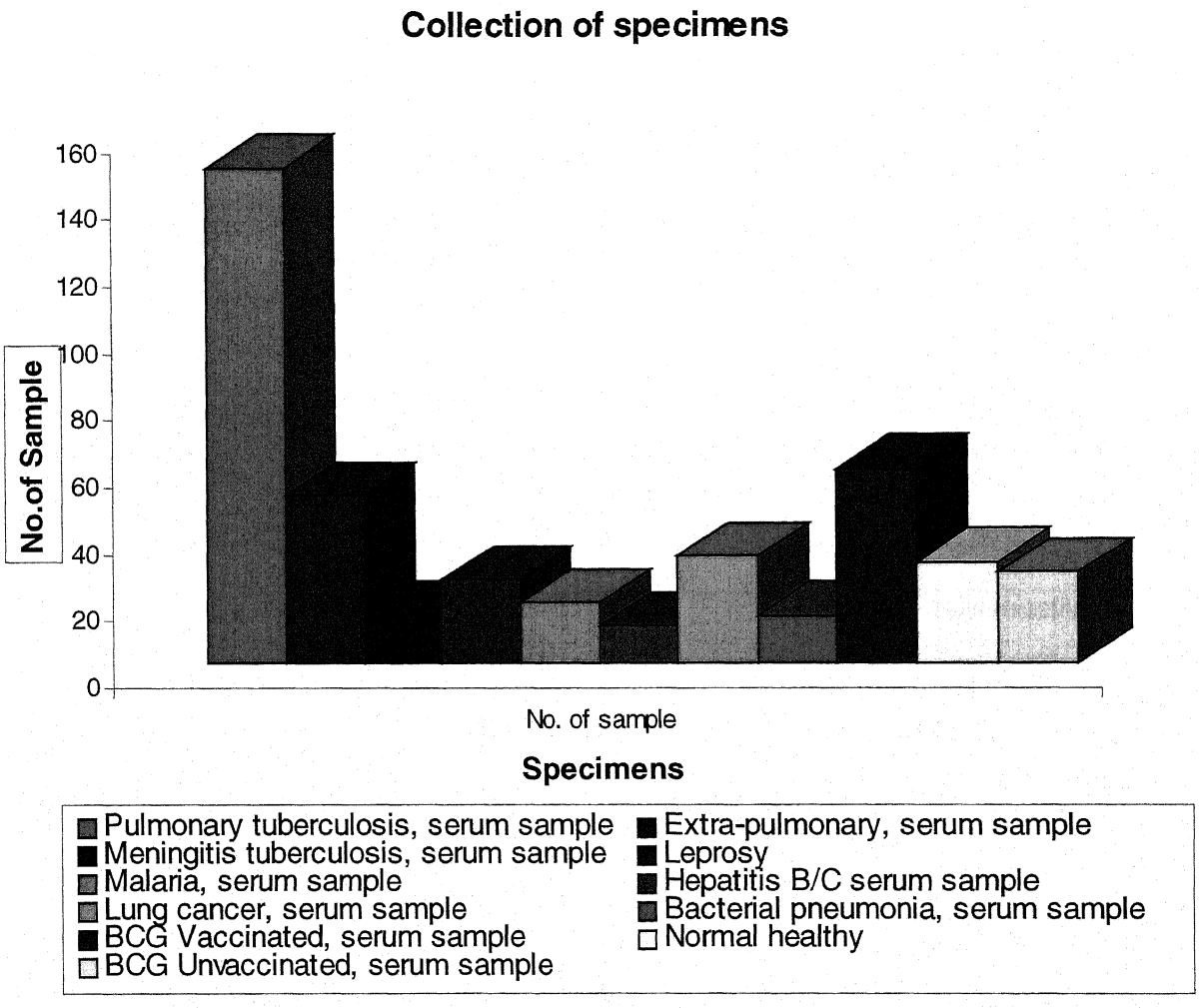

Figure: 39 Collection of defferent patient's samples from various sources.

The results of ELISA using cocktail antigen for 427 patients indicated following 139 subjects from total of 148 pulmonary subjects were positive, providing sensitivity of 94.27%. Similarly, out of 50 extra pulmonary patients, 46 were identified positive giving a sensitivity of 92.59%. Sensitivity for meningeal tuberculosis was found to be 60.86%. (Table-XXII and Figure-40).

Table- XXII

Evaluation of Cocktail of 'secretory protein Ag' with ELISA test

S. No.	Group	No. of Serum sample	Cocktail of Secretory protein Ag, ELISA Test		Reactivity
			+ Ve	-Ve	
1	Pulmonary tuberculosis	148	139	09	94.27%
2	Extra-pulmonary tuberculosis	50	46	04	92.59%
3	Meningitis tuberculosis	14	05	09	60.86%
4	Malaria	18	00	18	Non-reactive
5	Leprosy	25	02	23	92.59% specificity
6	Hepatitis B/C	11	01	10	Cross-reactive
7	Lung cancer	32	2	30	Cross-reactiv
8	Bacterial pneumonia	18	1	17	Cross-reactive
9	Normal healthy	30	30	30	Non-reactive
10	BCG Vaccinated	58	01	57	Cross-reactive
11	BCG Unvaccinated (Normal sample)	27	2	25	Cross-reactive

Sensitivity and Specificity- (± 2-3%).

However, the results were negative for the sera obtained from patients (n=146) other than tuberculosis indicating the specificity (92.59%) of the antigens for *Mycobacterium tuberculosis* infections. Further attempts should be made to check this specificity with the *Mycobacterium genera*.

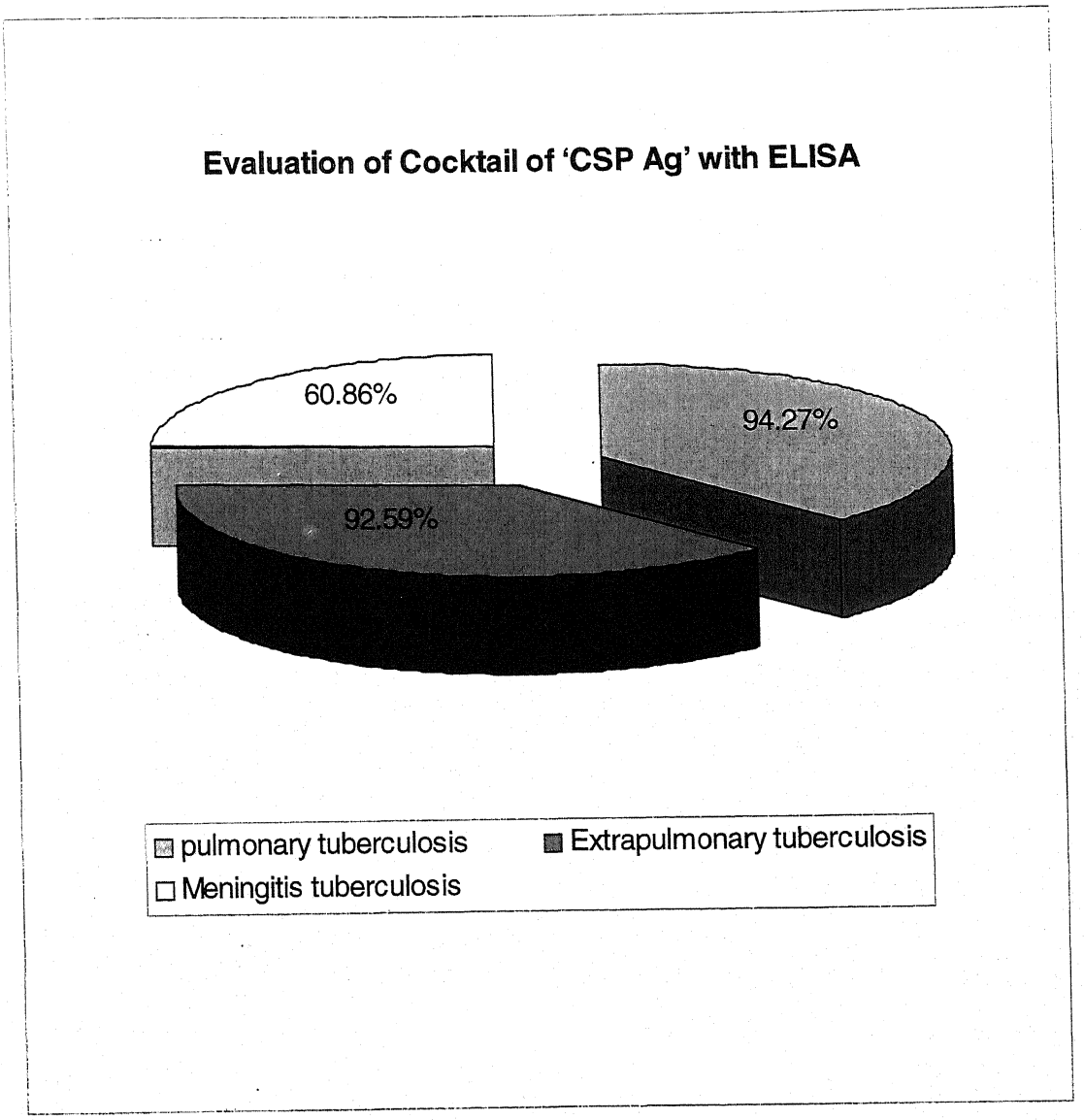

Figure: 40 Evaluation of cocktail of secretory proteins Ag with ELISA test.

The results of our study were also compared with a commercially available diagnostic kit containing cytosolic antigen A60 (Table- XIII and Figure- 41). It was observed that both the sensitivity, specificity of our combination of antigen and A60 was almost similar (±2-3%).

Table- XIII

Comparative evaluation of Cytosolic 'A60' Ag with ELISA test-

S. No.	Group	No. of Serum sample	Cytosolic 'A60' Ag, ELISA test +Ve	-ve	Reactivity
1	Pulmonary tuberculosis	148	127	21	87.57%
2	Extra-pulmonary tuberculosis	50	39	11	81.96%
3	Meningitis tuberculosis	14	04	10	58.33%
4	Leprosy	25	03	22	89.28% Specipicity
5	Malaria	18	00	18	Non-reactive
6	Hepatitis B/C	11	00	11	Non-reactive
7	Lung cancer	32	02	30	Cross-reactive
8	Bacterial pneumonia	18	00	18	Non-reactive
9	Normal healthy	30	30	30	Non-reactive
10	BCG Vaccinated	58	02	56	Croos-reactive
11	BCG Unvaccinated (Normal sample)	27	03	24	Cross-reactive

Sensitivity and Specificity- (± 2-3%).

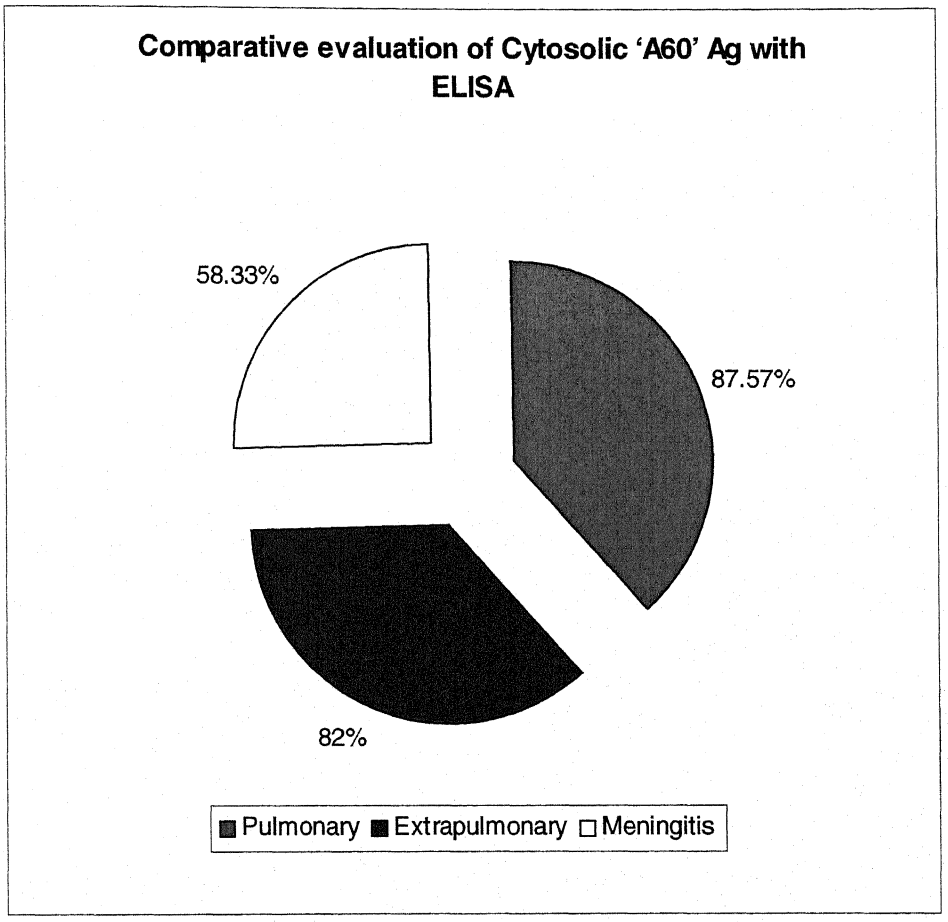

Figure: 41 Comparative evaluation of Cytosolic A60 ELISA Kit.

6

General Discussion

Tuberculosis is a disease of global importance. One-third of the world's population is estimated to be infected with *Mycobacterium tuberculosis* and eight million new cases of tuberculosis arise each year.

Since one third of the world's population carries *M. tuberculosis* in its latent form, and that 5% develop active disease during the first years of infection, it is estimated that 8 million new cases of tuberculosis and 3 million deaths occur each year (Dye, 1999 and WHO, 2004; Stefan and Andrew, 2005). Furthermore, the risk of reactivation increases as a consequence of associated pathologies, immunosuppressive therapy, malnutrition and, mainly coinfection with human immunodeficiency virus (HIV) (Rook GAW and Pando, 1996). Identification of these individuals is crucial for the control of disease transmission and elimination of tuberculosis.

The ability of a protein to detect antibodies present during subclinical disease is as important as the sensitivity of a protein in detecting antibodies formed during active tuberculosis, since therapy for latent tuberculosis infection can prevent the development of active disease.

Several antigens of *M. tuberculosis* have been found to be useful in the serodiagnosis of clinical disease (Bisen *et al.,* 2003; Garg *et al.*, 2003; Garg *et al.,* 2004), Traditional methods (smear and culture) are simpler and less expensive than the new molecular diagnostic tests such as PCR that are based on amplification of nucleic acid. Serological methods seem to be the ideal choice and, thus many *Mycobacterial* antigens like cellular extracts, proteins, (Arikan *et al.,* 1998) polysaccharides, DNA, RNA (Bisen *et al.,* 2003;

Garg *et al.*, 2003; Garg *et al.*, 2004) glycolipids (Julian *et al.*, 2002, Papa *et al.*, 1989) and other biomolecules have been evaluated. (Tiwari *et al.*, 2005).

It is evident from several studies that resistance against tuberculosis is mediated by arm of the immune system. Therefore, the antigen for vaccine development should be selected on its ability to induce the cell mediated immune response. In past, attempts have been made to investigate the immunogenic potential of different components of *Mycobacterium.* Several components *viz. Mycobacterial* cell wall, Heat shock proteins, ribosome/RNA and mannosides have been evaluated for their immuno protective potential (Mehta, 1988; Hetzel *et al.*, 1998; Oliver *et al.*, 2000; Gennaro, 2000; Leander *et al.*, 2005; Kwasi *et al.*, 2005).

Recently, the attention has been focused on secretory protein antigens of *Mycobacterium,* which are synthesized by the actively growing *M. tuberculosis* culture and to induce the desired immune response (Andersen *et al.*, 1991; Anderson, 1994; Kamath *et al., 1999;* Sonnenberg and Belisle, 1997; Karin *et al.*, 1998; Kanaujia *et al.*, 2004; Spencer *et al.*, 2004; Young *et al.*, 2004; Sable *et al.*, 2005). These proteins have also been termed as culture filtrate proteins (CFP) and known to elicit strong immune reactions in humans and animals infected with *M. tuberculosis / M. bovis* (Anderson *et al.*, 1991; Orme *et al.*, 1992; Romain *et al.*, 1993; Anderson, 1994).

The secretory proteins have been demonstrated to be strongly recognized by T-cells isolated from human (Tuberculosis) TB patients (Orme, 1988a) as well as mice and cattle experimentally infected with tuberculosis (Anderson and

Heron, 1993; Pollock and Anderson, 1997; Kanaujia *et al.,* 2004; Lanbo *et al.,* 2004). Experimental work in animal models suggest that both CD_4^+ and CD_8^+ T-cells are required for optimal protection against tubercle bacillus (Orme *et al.,* 1992; Bonato *et al.*, 1998; Flynn *et al.*, 1992; Pais *et al.*, 1998; Stefan and Andrew, 2005).

Secretory proteins, for comparison, are broadly recognized early during disease development in different species infected with *M. tuberculosis* (Anderson *et al.,* 1995; Brandt *et al.,* 1996; Pollock and Andersen, 1997; Kanaujia *et al.,* 2003).

They have discriminated TB patients from both BCG vaccinated and *M. avium* patients and have therefore been suggested as candidates for *in vitro* TB diagnosis (Lein *et al.,* 1999; Ulrichs *et al.,* 1998).

A combination of secretory protein (CFP) antigens selected by our approach can provide clues, which would allow the host immune responses to be better understood. This approach had also been successful in bringing about selection and use of antigens, which may be utilized for serological based diagnostic assay for tuberculosis and subunit vaccine.

Our efforts were intended to focus on secretory protein antigens, which can be suitable for developing a reliable diagnostic test for tuberculosis and utilization of subunit vaccine (protective efficacy). Such antigens, if found, can be easily incorporated into Enzyme Linked Immunoassays or all those related assays which utilize antibody antigen interactions as a marker of diagnosis, as has been utilized by others (Hirayama *et al.,* 2005).

Since the ELISA technique has the advantage of being a semi automated procedure with the capacity to analyze multiple samples and the equipment needed is available in many hospitals even in the developing countries of the world. As opposed to other infections, the use of ELISA in the diagnosis of tuberculosis has never been widely implemented due to problems with both sensitivity as well as specificity. For developing such assays, it was desirable to find out and purify Secretory protein (CFP.) antigens, which may specifically diagnose active tuberculosis sufferers.

The present study was investigated to examine the immuno diagnosis and immunoprotective activity of the secretory proteins of *Mycobacterium tuberculosis* $H_{37}Rv$. The Secretory proteins were isolated from logarithmic phase culture (5 week old) of *Mycobacterium tuberculosis* $H_{37}Rv$ without shaking at 37°C (Nagai *et al.,* 1991). It has been earlier demonstrated that the cultivation time, growth of bacteria can be examined by microscopy (Figure-9) and spectrophotometer at A_{580} (Figure- 11). Protein secretion was monitored through the protein estimation of culture medium by at A_{280} (Figure-12) and the profile of proteins released into the culture supernatants of the tubercle bacilli by SDS-PAGE.

The major secretory proteins were purified by following the method of Nagai *et al.* (1991). For initial fraction of major secretory proteins, DEAE sepharose CL-6b anion exchange column equilibrated with Tris-HCl was used. The five proteins (6 kDa, 26, 30,38 and 64 kDa) in peak I, II, III, IV, V, and VI were virtually obtained in pure form in this column and characterized by SDS PAGE

and Tricine SDS PAGE (low molecular weight proteins); again the individual protein band were then eluted from the gel. The reactivity of secretory protein antigens were confirmed by western blotting with positive tuberculosis patient's sera and ELISA.

We selected for the study, secretory protein antigens with molecular weight of 6 kDa, 26 kDa, 30 kDa, 38 kDa and 64 kDa for serodiagnosis and protective efficacy.

We used Western blot because it is a method that provides a safe reading and interpretation of the results when duly standardized. In the present study, we produced industrial lots of antigen fractions that were assessed for reproducibility with control serum samples collected from patients with confirmed pulmonary tuberculosis and healthy individuals. Compared to molecular methods, Western blot has the advantage of being easy to perform, considering that small laboratories are better equipped for the execution of serological tests, detection immuno reactivity than of molecular tests. The stability of the nitrocellulose strips, that can be stored for more than 12 months, permits the execution of the test in laboratories with small routines. The Western blot method has been widely used to confirm serologic results and for the identification of the immune response against different protein fractions of viruses, fungi, and parasites (Jackett *et al.,* 1988).

Studies employing Western blot analysis have shown that *M. tuberculosis* secretory protein antigens in the range of 64, 38, 30, 26 and 06 kDa frequently react with tuberculosis positive serum (Figure-18 to 23).

Further, We also used Enzyme-Linked Immunosorbent Assay (ELISA) because it is a powerful method in estimating ng/ml to pg/ml ordered materials in the solution, such as serum, urine and culture supernatant. There are different kinds of ELISA employed for different conditions. In these studies, since the antigens/ antigenic epitopes were developed and very well characterized, indirect ELISA was found most suitable for detecting their corresponding antibodies in the solutions.

1. In indirect ELISA, if solutions contain antibodies to the antigenic epitopes, those antibodies bind to the corresponding Secretory protein antigens on the plate. Unbound antibodies are washed out and followed by reaction with anti-human immunoglobulin coupled to an enzyme (e. g. Horse, radish Peroxidase). This is the second antibody that binds to human antibodies captured on the plate. Unbound enzyme coupled immunoglobulins are washed out and are followed by reaction which further changes the color of chromogen. The enzymatic reaction is quenched after an optimum time period by denaturing the enzyme. The color of final product is measured calorimetrically and interpreted.

When considering the binding capacity of adsorbent plastic surfaces for biomacro-molecules, it is essential to distinguish between the total amount of molecules that can be bound to the surfaces and the amount that can be bound and still remain biologically active. Both quantities are very much dependent on the nature of the molecules and the character of the surface. The adsorption of molecules to a polystyrene surface is due to inter-molecular

attraction forces (van der Waals forces), to be distinguished from true chemical bonds, i. e. covalent bonds (through electron share) and ionic bonds (through stoichiometric charges of opposite signs). There are four main types of possible bonds between macromolecules.

a) True chemical bonds are represented by a covalent disulphide bond .

b) Ionic bond between a carboxyl ion and an amino ion.

c) Van der Waals mediated bonds are represented by a hydrogen bond between two dipoles and an alternating polarity bond between hydrocarbon residues protruding from the macromolecules backbones, Where the encircled area indicates a water-deprived zone.

There are two different types of absorbent polystyrene surfaces that are available from Nunc.

- POLYSORP surface
- MAXISORP surface

PolySorp Predominantly presents hydrophobic groups, MaxiSorp has in addition many hydrophilic groups, which results in a fine patchwork of hydrophobic and hydrophilic binding sites. In this study MaxiSorp surface was used for facilitation of the absorption of hydrophilic synthetic epitopes, because not only can this surface compete with the water molecules for binding the macromolecules by hydrogen bonds, but the molecules can also be captured from a much longer distance by the long-range hydrogen bond forces for establishment of both hydrogen bonds and eventually hydrophobic bonds. However, with MaxiSorp peptide epitopes binding events are more

likely to occur, which means that adequate incubation conditions are easier to establish.

As mentioned above, van der Waal mediated bonds are relatively weak, therefore, they may be insufficient for stable bonding when they are few in number, i.e. when the molecules are small. For binding of small molecules, strong chemical bonds are needed. Ionic bonds would not do, because they normally dissociate in aqueous solution, leaving covalent bonds as the only possibility for direct, stable binding of small molecules e. g. peptides. However, this difficulty may be overcome by using small molecules linked to (indifferent) carrier macromolecules as supported by this study.

Antigen coating efficiency is dependent on immobilization pH (Coating buffer), ionic strength, immobilization time (incubation period), concentration of antigens, and temperature of immobilization.

Antigens showed maximum absorption on solid phase at pH 7.2 whereas cocktail of antigens showed maximum adsorption at pH 9.2.Since there was no much difference in the adsorption between pH 9.2 and 7.2. Hence, the common pH was selected for the immobilization of cocktail of epitopes.

All the antigens showed maximum absorption on solid phase between 10 mM to 50 mM but reactivity fall down at 100 mM. The saturation of the adsorption of all the epitopes on solid phase took place within 18 hours.

Investigation of immobilization of antigens with the variation in the concentration revealed that there is a rise in the concentration of all the protein antigens from 2.5 ug/ well to 7.5 ug/ well and then after, there was no effect of higher antigen concentration on immobilization as evident by the experimental data.

Work on the immobilization time at 37°C showed that there was rise in the adsorption of the antigens on the surface up till 120 minutes followed by saturation. Although optimum adsorption took place in 120 minutes, but it was advisable to prolong the incubation period for another 120 minutes.

Blocking agents are essentially to be used in ELISA for blocking possible excess solid surface after coating with antigen to avoid unspecific immobilization of succeeding reactants. One reason for using a true blocking agent would be to substitute detergent for blocking. If detergent is present during incubation with secondary reactants, it might in some way interfere with the immunologic specificities or cause unspecific immobilization of the reactants (Esser, 1990). If detergent is present during wash after secondary reactants, possible weak immunologic affinities might be broken by the washing activity of the detergent. Another reason for using a blocking agent would be to stabilize the immobilized antigens of coated surfaces. Typical blocking agents would be an indifferent macromolecule, large enough to establish a stable attachment to the surface, yet small enough to find its way between antigens.

Bovine serum albumin (BSA), of MW 67,000 is commonly used as a blocking agent. Also the more heterogeneous casein is often used and may be more effective than BSA (Pratt and Roser, 1989; Vogt *et al.,* 1987). In this study Casein digest, hydrolysate was found to be the best blocking agent amongst all the three blocking agents studied. Casein digest, hydrolysate was found better than BSA (Table-III, Figure- 24), which was further found better than normal rabbit serum.

Casein digest, hydrolysate stood to be the best blocking agent amongst all the three studies due to its following features:

1. Due to its heterogeneous nature (different masses of peptides) it blocks the inter-epitope space and unimmobilized space on the solid surfaces more firmly than BSA and normal rabbit serum.
2. It also has exerted lesser effect on the stearic hindrance in antigen-antibody reaction.
3. It also has lesser effect on the shielding epitopes.

In this regards 0.1% of casein digest, hydrolysate, in phosphate buffered, 100mM, pH 7.2 was found sufficient enough to serve the purpose of blocking.

Assay employing the cocktail of selected antigen was evaluated using the specific antibodies to the antigens to validate the immobilization of particular antigens epitope and found that all the epitopes subjected for coating were immobilized optically. This assay differentiated the reactive and non-reactive samples.

Amongst all the reagents in ELISA, anti IgG-HRP conjugate, solid phase immobilized antigens and the enzyme substrate-chromogen (TMB-H_2O_2) are very critical, hence an effort was made to stabilize them. In the conjugate, HRP is very prone to peroxidation that was protected by a reducing agent (TMB). Protein part of the conjugate was protected from deterioration by protein stabilizers (casein digest, hydrolysate), preservatives (thiol containing compounds), surfactant (Tween – 20) and antibacterial compounds.

To determine the antibody response to secretory protein (CFP) antigens in tuberculosis patients, (Imaz and Zerbini, 2000). We determined the specific response to the 30kDa and 6kDa antigens. A possible explanation for this phenomenon may be the intense stimulation of the immune response by antigens released by bacteria, and / or by the release of antibodies that were previously part of immune complexes.

The response to the 30 or 6kDa secretory protein antigens was reactive in TB patients than in contacts or healthy subjects. The presence of these antibodies in tuberculosis patients has been associated with a more favorable prognosis or with spontaneously cured tuberculosis. Thus, the presence of these antibodies in contacts may suggest a tuberculosis infection that is not clinically evident.

It has been investigated that the antibody response against antigens in the 6kDa secretory proteins is suitable for diagnostic purposes as well as protective efficacy. The antigen possesses various specific T-cell epitopes that induce a cellular response and lead to increased interferon gamma production in patients with sub clinical or active tuberculosis but not in

unexposed healthy individuals (Arend *et al.,* 2001). This cell-mediated response has also been associated with increased risk of disease (Doherty *et al.,* 2002). A recent study showed that the humoral response to ESAT-6 may be associated with inactive tuberculosis but not with active tuberculosis (Silva *et al.,* 2003).

These reactivity profiles showed sensitivity similar to that observed by other investigators. However, in the general population, especially in developing countries, with a high prevalence of *M. tuberculosis* infection, there are a percentage of persons who do not present clinical signs and symptoms of tuberculosis and who must be identified and treated. For this purpose, a highly specific test is needed in order to detect suspected cases.

Earlier studies have suggested that the humoral response to *M. tuberculosis* antigens was increased in patients with an inadequate cell-mediated response to the same antigens (Bhantnagar *et al.,* 1977) and in patients that showed a strong humoral response after the infection led to the development of active tuberculosis (David *et al.,* 1992). This evidence, together with a recent study that evaluated the humoral response to recombinant *M. tuberculosis* antigens, 30kDa and 6kDa showing association of the latter two antigens with risk factors for future active, but not current disease suggest the possibility of identifying the subset of persons with latent tuberculosis infection who may be at high risk to develop active disease.

Since significant geographical variation in antibody titer to some antigens exists, heterogeneous recognition of antigens by serum antibodies in tuberculosis can result from multiple factors (Lyashchenko *et al.,* 1998).

The humoral response mainly against antigens of mass 30kDa, 6 kDa and cocktail seems to be important for the detection of latent tuberculosis.

Immunoreactivity of Purified Secretory Proteins

Total culture filtrate proteins (CFP) when administered as CFP-IFA complexes elicited both humoral and cellular immune response (Table- X and Figure-31) to all the five purified secretory proteins. CFP elicited significant antibody response to all the five antigens throughout the observation period (fourth week). The optimal response was obtained at third week (Table-X and Figure-31) for 6kDa, 38kDa, 64kDa and CFP secretory proteins. The induction of high levels of antibodies to the various secretory proteins of *Mycobacteria* has been reported earlier by few workers in the animal and humans infected with *M. tuberculosis* (Pessolani *et at.,* 1989; Huygen *et al.,* 1996; Lanbo *et al.,* 2004) and in animals immunized with the culture filtrate proteins of *Mycobacteria* (Daugelet *et al.,* 1992; Andersen, 1994).

Besides the humoral response, enhanced sensitization, implying the activation of T-lymphocyte by the CFP against all the five antigens was seen by *in vitro* T-lymphocyte proliferation assay (Table-XI and Figure- 32). The proliferation activity was observed with all the purified proteins in the lymphocytes obtained from immunized animals. However, the maximum activation T-lymphocytes was seen with 6kDa secretory antigen throughout the time period with peak activity in third week p. im. as compared to the other antigen . This observation has been in concurrence with the reports of other workers that the secretory proteins are (Orme and Collins, 1983; Pal and Horwitz, 1992;

Andersen *et al.,* 1991b; Andersen. 1994) the major targets of T-lymphocytes. This also explains the superiority of live BCG vaccine over the killed vaccine in evoking the strong immune response and protection (Smith, 1985) possibly due to the secretory proteins, which may be largely responsible for its protective efficacy. Further, the observation that 30 and 6kDa secretory proteins being the most immunoreactive protein of the five secretory proteins is in agreement with the observation that the secretory proteins of immune T-lymphocytes (Andersen *et al.,* 1991; Hubbard *et al.,* 1992; Daugelat *et al.,* 1992; Andersen, 1994). Detailed investigations of immune responses were carried out with 6kDa secretory protein since, it was the most immunoreactive and major secretory protein as compared to other purified secretory proteins of *M. tuberculosis* H_{37}Rv (Table-X, XI and Figure- 31,32).

Immungenicity of 6 kDa Secretory Protein Complexed with IFA

Different concentrations of 6kDa antigen (75 ug, 37.5 ug and 18.75 ug) when administered as 6kDa IFA complexes in animals elicited cellular and humoral responses (Table-XII and Figure- 33). An enhanced antibody level was demonstrated in all the groups of mice immunized with different concentrations of 6kDa antigen throughout the time period attainting its peak level in 4^{th} week (Table-XII and Figure- 33). Similarly, there was significant T-lymphocyte proliferation in 6kDa-IFA immunized animals with different concentration of antigen. The pronounced T-lymphocyte proliferation activity of 6kDa protein in animals immunized with total culture filtrate might be due to the presence of several proteins in culture filtrate which perhaps influence the immunoreactivity of 6kDa secretory protein. Furthermore, the immune

responses in present study (cellular and humoral) were found to be dose depended demonstration higher activation of immune cells in animals immunized with highest concentration of antigen (75µg). Similar observation has been reported by Andersen (1994) that the increase in the doses of immunogens from 10-250 µg was associated with the increase of more than 100ug resulted in the shift of immune response from Th1 towards Th2 type, thus emphasizing that the dose of antigen should be carefully standardized to obtain the desired immune response.

Furthermore, the immune response induced with 75ug concentration of the antigen was higher (Table-XIII and Figure- 34) than observed in BCG vaccinated mice. Our observations are in agreement with earlier reports that the immunization with pure protein or combination of proteins induces better immune responses compared to that induced by the whole organism (Pal and Horwitz, 1992; Andersen, 1994; Ingrid *et al.,* 2000; Kanaujia *et al.,* 2004; Stefan *et al.,* 2005).

Protective immunity in tuberculosis and the diseases caused by intracellular pathogens are mediated by specifically activated T-lymphocytes (Orme and Collins 1983; Blander and Horwitz, 1993). It is believed that the $CD4^+$ T-lymphocytes act as primary effecter cell population which is responsible for the induction of acquired resistance against the disease (Muller *et al.,* 1987; Orme, 1988a). The $CD4^+$ T- lymphocytes can be further differentiated into two major subsets Th1 and Th2 on the basis of cytokine profile secreted by the activated $CD4^+$ T- lymphocytes (Mossaman *et al.,* 1986; Stefan *et al.,* 2005).

These findings suggest that 6kDa secretory proteins induce the activation of Th1 type of T- lymphocytes.

Therefore, it is concluded that 6-kDa secretory protein of *M. tuberculosis* $H_{37}Rv$ complexes with IFA elicited strong cell mediated and humoral immune response, which was skewed towards Th1 type of T- lymphocyte activation.

Protection Conferred by 6 kDa antigen complexed in IFA

BCG is the most widely used live vaccine in the prophylaxis against tuberculosis, the efficacy of which varies between 0-80% (Baily, 1990; Fine, 1989). Hence, efforts are being made to identify the immunoprophylactic components of *Mycobacteria*. Amongst the various *Mycobacterial* components, secretory proteins have been proposed to be important antigens for the development of subunit vaccine. *Mycobacterial* secretory proteins released by actively growing cells are established that 6 kDa secretory protein of *M. tuberculosis* $H_{37}Rv$ induced strong humoral and cell mediated immune responses when complexed in IFA, the prophylactic significance of this protein was explored. In experimental infections for the evaluation of new prophylactic agents, responses are strongly influenced by the choice of animals, route of administration of antigen and size of challenge dose (Ribi *et al.,* 1965; Newman and Powll, 1995). The choice of mouse as an experimental model is justified, as it is cost effective and relatively easy to handle. It also has the advantage of well established genetic and immunological markers which allow detailed analysis of immune reactions during immunization and infection (Harboe *et al.,* 1986). Similarly, the

immunization route used to deliver the subunit vaccines can dramatically influences the type of immune response generated (Fadda *et al.,* 1998). The choice of peripheral route for immunization using IFA by us in present study appears to be justified as the inoculation of oil treated components leads to graulomatous response in animals (Barclay *et al.,* 1967), also the LD_{50} of *M. tuberculosis* $H_{37}Rv$ was chosen because massive i. v. challenge with virulent *Mycobacteria* leads to a rapid response which masks the protective efficacy of vaccine.

The immunization of animals with different concentrations of 6kDa secretory protein complexes in IFA resulted in significantly higher percent survival (Table-XIV, XV and Figure-35) against an intravenous challenge with LD_{50} of *M. tuberculosis* $H_{37}Rv$ as compared to unimmunized animals. Moreover, the protection induced was found to be dose depended which was maximum (100%) in mice immunized with 75µg concentration of the antigen, again emphasizing the effect of dose the immunogens used to mediate desired immune response and protection. Since the mechanism of immunoprotection has been observed to be T- cell mediated (Orme and Collins, 1983; Andersen, 1994; Spencer *et al.,* 2004; Lanbo *et al.,* 2004; Sable *et al.,* 2005) the dose dependent increase in the protective efficacy is explainable.

Further, the immunization with 6kDa antigen demonstrated a significant percent survival, which was better than that observed in BCG vaccinated mice (72.7%) (Table- XV, XVI and Figure -35) and 45% in control groups, challenged at the peak of immune response with LD_{50} of *M. tuberculosis*

$H_{37}Rv$. Increased survival rates of animals actually reflects the retardation in the rate of multiplication of *tubercle bacilli* (Youmans *et al.*, 1976). Therefore, viable counts in the infected organs were enumerated. Immunized mice demonstrated a significant decrease in bacterial load thirty days after the challenge (Table-VI) in lungs, liver and spleen as compared to controls. This further supports the observed higher percent survival and also satisfies the criterion for the observed higher percent of vaccine (s) against tuberculosis (Weigeshoua *et al.,* 1970). The decrease in CFU's recovered from liver and lung in 6kDa immunized was better than that obtained with BCG vaccinated mice. The induced protection by 6kDa antigen was also better than that obtained with BCG vaccine by other workers (Orme, 1988a; Andersen, 1994; Roberts *et al.,* 1995). These findings are further substantiated by the earlier reports (Hubbared *et al.,* 1992; Pal and Horwitz, 1992; Andersen, 1994; Howitz *et al.,* 1995) where in the protective effect of the secretory proteins has been demonstrated to be comparable to BCG vaccine. Interesting, Andersen (1994) had shown the maximum immune reactivity in the region of 6kDa in ST-CF immunized and BCG vaccinated mice the major immunoprotective antigen of *M. tuberculosis* $H_{37}Rv$.

Since, during the initial stages of immunization with antigen in Incomplete Freund's adjuvant (IFA), the nonspecific inflammatory responses might have affected the degree of protection and immune response, the protection efficacy was determined after 1^{st} and 4^{th} weeks of immunization to ensure that the nonspecific response due to antigen and IFA are diminished. The 6kDa-IFA immunized mice challenged 7 and 30.Days post immunization, demonstrated a percent survival and at thirty days post challenge only 30% of

the control mice survived the challenge. On the other hand, the mice immunized with varying concentration of the antigen demonstrated no mortality till two weeks post challenge. However, the onset of mortality occurred during third week post challenge with 20% 30%and 40% mortality in animals immunized with 75µg, 37.5µg, 18.75µg of the antigen respectively and 20% mortality was also observed in animals vaccinated with BCG vaccine. In the fourth week an increased mortality (50% & 60%) was observed in the lower two doses of 6kDa immunized animals. Mortality was also observed in 75µg protein (25%) and BCG vaccinated (30%) animals.

At the end of 30 days observation, 75% survival was seen in animals immunized with 75µg concentration of 6kDa protein that was similar to survival (70%) afforded by BCG vaccine.

Thus indicating that protection imparted by 6kDa secretory antigen is mediated by specifically activated T-lymphocytes. The observed decline in protective immunity to virulent challenge of *M. tuberculosis* $H_{37}Rv$ might be due to the protective immunity and heightened state of memory (Sprent, 1993) however, as the protection afford by 6kDa-IFA immunization during 3rd week remained stable indicates the induction of memory T-lymphocytes. The maintenance of stable state of immunity by BCG vaccine throughout the observations period could be due to the continuous stimulus provided by the secreted proteins released by actively growing cells. Our observations are supported by the findings (Pal and Howitz, 1992; Andersen, 1994; Roberts *et al.,* 1995; Horwitz *et al.,* 2000) that culture filtrate proteins of *Mycobacteria*

confer protective immunity, equivalent to the BCG vaccine. Further, the protection imparted by culture filtrate proteins was shown to be long lasting and mediated by T- lymphocytes (Andersen *et al.,* 1994).

Thus our results also support the emerging hypothesis that secretory proteins are the key immunoprotective molecules capable of generating a strong cell mediated immune response leading to an efficient acquired resistance against virulent *M. tuberculosis* $H_{37}Rv$ and other intracellular pathogens.

Immunoprotective Activity of 6kDa Secretory Protein Entrapped in Liposome

Previous observation about 6kDa secretory protein (CFP) of *M. tuberculosis* $H_{37}Rv$ complex with FIA could induce both cellular and humoral responses. Furthermore, immunization with 6kDa-FIA was able to confer significant short and long-term protection against tuberculosis. Further, studies were carried out to evaluate the immnuoprotective behavior of 6kDa secretory protein using liposome as adjutants.

Although, FIA is the most commonly used adjuvant for experimental studies, it is far from idea with respect to human use because of severe side effects associated with it (Freund, 1956; Gupta *et al.,* 1993). Thus, an adjuvant, which does not cause any adverse reaction, is required for development of alternate subunit vaccine. Recently, the use of a large number of adjuvants (FIA, RIBI adjuvant, DDA, ISCOMS, Saponins microshperes i. e. PLG and Liposomes) have been reported to be potent inducers of cell mediated immunity has emphasized the decisive influence of adjuvant on vaccine

efficacy. Among the different adjuvant systems, liposomes have been viewed as an alternative to alum based (only adjuvant licensed for human use) adjuvant. In earlier studies, the phosphatidylcholine (PC) liposomes have been shown to be promising adjuvants for proteins antigens (Lawman *et al.,* 1981; Pimn and Baldwin, 1984; Pancholi *et al.,* 1989; Chugh and Khuller, 1993). Further, incorporation of charge in PC liposomes, method of preparation and addition of other adjuvants such as Lipid A, muramyl dipeptide, alums, has been reported to boost the adjuvanticity of liposomes (Fries *et al.,* 1992; Gupta *et al.,* 1993).

In this study, immunogenic role of 6kDa secretory protein entrapped in liposomes has been investigated. Liposomes used in the study were prepared by Freeze-thaw method (Mayer *et al.,* 1986) containing phosphatidylglycerol (PG), cholesterol (Ch) and adsorbed on to alums. Percent entrapment of 6kDa secretory antigen in liposomes ranged from 52-54%, which was comparable to 45-50% entrapment, observed with protein antigens employing the same method of preparation (Meyer *et al.,* 1986).

Immunization with 6kDa antigen encapsulated in liposomes, included significant humoral and cell mediated immune responses as determined by ELISA and T-lymphocyte proliferation assay. The augmentation of T and B lymphocyte mediated immune response is in accordance with earlier reports in which liposomes have been used as an adjuvant for *Mycobacterial* antigens (Pancholi *et at.,* 1989, Chugh and Khuller 1993; Andersen, 1994) and membrane antigens of Leishmania donovani and Entamoeba histolytica (Vinayak *et at.,* 1987; Russell and Alexender, 1998). It is widely believed that

the process of immunological presentation of protein antigens to the immune system involves initial processing of the antigen by professional antigen presenting cells such as macrophages and dendritic cells (Unaue and Cerottini, 1989). The ability of liposomes encapsulated antigens to enhance the immune response is due to the natural targeting of liposomes to macrophages (Shek and Sabistan, 1982; Van-Rooijen and Ven Nieuwmegen, 1983a; Beatty *et at.,* 1984). Activated macrophages have been shown to play a major role in defense against infectious organism in *vivo* and *in vitro* (Kirsh and Poste, 1986).

The immune response (cellular and humoral) elicited by the 6 kDa secretory protein entrapped in liposomes were similar (Table-XII, VIII and Figure-36, 37) to that elicited by 6 kDa-FIA and BCG vaccine, thus indicating that liposomes can be effectively used to generate strong immune response by the entrapped antigen. Immunization with 6 kDa secretory protein entrapped in liposomes resulted in increased resistance to intravenous challenge with LD_{50} of *M. tuberculosis* $H_{37}Rv$ 4th weeks post immunization as monitored by survival rates and enumeration of viable tubercle bacilli in the infected organs (spleen, liver and lung). Immunized animals with 6kD-Lip and BCG showed considerably higher ($p<0.001$) survival rates (76%), (72%) as a compared to control animals (32%) on 30 days post challenge (Table-XIX and Figure - 38).

There was a generalized drop in the number of bacteria isolated from spleen, lungs and liver (Table- XX) as compared to control mice indicated that immunization with 6kDa-Lip ensure better clearance of the microorganism from the three organs (liver, lungs and spleen).

Our findings that liposomes could be effectively used to elicit the desired immune response and confer significant protection in the immunized animals are similar to the observation of other workers who have demonstrated increased immune response (Vinayak *et al.,* 1987; Russell and Alexender, 1998; Pancholi *et al.,* 1989; Chugh and Khuller, 1993) with the liposomal encapsulated antigen. The immunogenicity of the liposomal entrapped antigen used in the study was comparable to that induced by the incorporation of the culture filtrate / extracellular proteins of *Mycobacteria* in different adjuvant system such as Syntax (SAF1), DDA, RIBI adjuvant, and micro sphere (PLG) particles (Andersen, 1994; Horwitz *et al.,* 1995; Vordermeier *et al.,* 1996). These adjuvants have been reported to augments, potent cell meditated immunity with secretion of IFN-γ and generation of IgG a antibodies. DDA, SAF1 and liposomes entrapped antigens have also been shown to impart protection against the disease in animal model (Andersen, 1994; Horwitz *et al.,* 1995). Some recent reports suggest that saponin adjuvant (such as ISCOMS) stimulate a mixed response skewed toward a Th1 response characterized by production of IFN-γ and some IL-5 from primed T cells.

Thus, the use of liposomes to deliver the antigen to generate desired cellular immune response and impart protection appears to be better, as liposomes have the ability to load a large variety of molecules into them regardless of their size, solubility and charge characteristics (Gregoriadis, 1990). The extensive knowledge of liposomal properties, behavior in *vivo*, the variety of ways of controlling such behavior and their non-toxic nature supports further

efforts towards the application of the liposomes as immunological adjuvant and vaccine carriers.

Serodiagnostic potential of cocktail of antigens

Developed ELISA based serodiagnostic test using a cocktail of all five proteins was used to enhance the sensitivity of the assay and attempts were also made to check the specificity of the cocktail.

Out of 148 pulmonary patients, 139 were positive, providing sensitivity of 94.27%. Similarly, out of 50 extra pulmonary patients, 46 were identified positive giving a sensitivity of 92.59%. Sensitivity for meningeal tuberculosis was found to be 60. 86% (Table-XXII and Figure- 40).

The results of our study were also compared with a commercially available diagnostic kit containing cytosolic antigen A60 (Table –XXIII and Figure- 41). It was observed that both the sensitivity, specificity of our combination of antigen and A60 was almost similar.

7

Summary and Conclusion

Tuberculosis (TB) an air borne communicable disease and one of the three World Health Organization (WHO) priority diseases is caused by transmission of aerosolized droplets of *M. tuberculosis*. It kills about 2 million people annually and the epidemic is spreading globally, assuming alarming proportion. Around 8 million people become infected with TB every year. Diagnosis of tuberculosis is primarily based on X-ray examination and / or sometimes on symptoms that are often confusing and misleading. While infectious cases are frequently missed, some people are mistakenly diagnosed with TB and inappropriately treated.

Further the prevention of disease presently is made through BCG vaccine administered at the time of birth. However this vaccine also has several limitations and its efficacy has been reported to vary from 0-80%. Hence a new safe and immunogenic vaccine against tuberculosis is highly desirable. In past, several attempts have been made to develop killed and live vaccines to protect against the disease however, till date there is no alternative to the BCG vaccine.

Hence, there is an urgent need to develop identifying the immunoprotective and immunodiagnostic antigens of *M. tuberculosis* for the development of effective vaccine and / or diagnostic tool.

The present study was designed to isolate and screen low molecular weight secretory proteins possessing potentials to activate the cellular immune

response and those having ability to induce high titer antibodies for the development of prophylactic and diagnostic tool.

Secretory protein(s) of *Mycobacterium* are synthesized by the actively growing *M. tuberculosis* cells and induce the significant immune response. These proteins have been demonstrated are recognized by T-cells isolated from human (Tuberculosis) TB patients as well as mice and cattle experimentally infected with tuberculosis. Experimental work in animal models suggests that these proteins activate both CD4+ and CD8+ T-cells.

Our efforts in the present study were intended to focus on secretory protein antigen, which can be suitable for developing a reliable diagnostic test for tuberculosis and utilization of subunit vaccine. Such antigens, if found, can be easily incorporated into Enzyme Linked Immunoassays or all those related assays which utilize antibody antigen interactions as a marker of diagnosis, as has been utilized by others. The ELISA technique has the advantage of being a semi-automated procedure with the capacity to analyze multiple samples and the equipment needed is available in many hospitals even in the developing countries of the world. As opposed to other infections, the use of ELISA in the diagnosis of tuberculosis has never been widely implemented due to problems with both sensitivity as well as specificity. For developing such assays, it was desirable to identify and purify Secretory protein (CFP) antigens, which may specifically diagnose active tuberculosis sufferers.

Purification of protein(s)

1. The Secretory proteins were isolated from logarithmic phase culture (5 week old) of *Mycobacterium tuberculosis* $H_{37}Rv$ without shaking at 37 °C.

2. Protein secretion was monitored through the protein estimation of culture medium by at A_{280} and the profile of proteins released into the culture supernatants of the tubercle bacilli by SDS-PAGE.

3. The major secretory proteins were purified with reference to the Nagai *et al* (1991) using DEAE sepharose CL-6b anion exchange column.

4. Five proteins (6.0, 26.0, 30.0, 38.0 and 64.0kDa) were obtained in purified form by column chromatography and SDS-PAGE. These proteins were selected for serology and cellular reactivity.

Humoral response

For measuring the humoral response and detection of antigens, indirect ELISA was used for this, the conditions for ELISA were optimized.

All five proteins were assayed by using sera from tuberculosis positive patients. These proteins demonstrated presence of antibodies in the serum however the quantity of some was lesser compared to the other as observed by western blot band intensity. The reactivity profiles showed is similar to that observed by several other investigators.

The humoral response against the five proteins individual and as a cocktail of five antigens was studied in sera of mice immunized with total culture filtrate proteins.

Linear increase in the antibody titer was observed up to four weeks of immunization. Out of the five antigens two antigens 64 kDa, and 38 kDa demonstrated significant higher level of antibodies against them in the sera of immunized animals.

Since low titer antibodies were also seen against other three proteins we prepared a cocktail of these five proteins and used them in combination for testing the presence of antibodies against them in sera of tuberculosis patients.

The results of serology by ELISA demonstrated that the present cocktail effectively detects the antibodies in the patient sample with increased sensitivity.

This combination (cocktail of five proteins) thus can be used to develop diagnostic tool for detection of antibodies however, further work is needed to establish the sensitivity and specificity of this combination in selectively identifying the *M. tuberculosis* complex in the sample

Cellular Immune Response

Cellular immune response against the five proteins in mice was determined by immunizing the animals (mice) with total culture filtrate proteins (CFP) administered as CFP-IFA complexes. Immune response was determined by measuring T-cell proliferation by these antigens.

Significant T-cell proliferation was observed by all the five antigens however maximum response against antigens having molecular weight of 30 and 6kDa.

Maximum T-cell activity was observed in the third week post immunization for 6 and 30kDa secretory proteins.

Further studies were undertaken to test the immunogenic potential of 6.0 kDa secretory protein for this animals were immunized with three different concentrations i. e. 75µg, 37.5µg and 18.75µg.

An enhanced T-cell Proliferation was observed in all the groups of mice immunized with different concentrations of 6kDa antigen throughout the study period attainting its peak level 4^{th} week p. im.

Furthermore, the immune response in present study was found to be dose dependent, demonstrating higher activation of immune cells in animals immunized with highest concentration of antigen (75µg).

Furthermore, the immune response induced with 75µg concentration of the antigen was higher than observed in BCG vaccinated mice. Our observations

are in agreement with earlier reports that the immunization with pure protein or combination of proteins induces better immune responses compared to that induced by the whole organism.

Protective potential of 6.0kDa protein

The immunization of animals with different concentrations of 6kDa secretory protein complexes in IFA resulted in significantly higher percent survival against an intravenous challenge with LD_{50} of *M. tuberculosis* $H_{37}Rv$ as compared to unimmunized animals.

At the end of 30 days observation, 75% survivals were animals immunized with 75µg concentration of 6kDa protein that was similar to survival (70%) afforded by BCG vaccine.

Moreover, the protection induced was found to be dose depended which was maximum (75%) in mice immunized with 75µg concentration of the antigen, again emphasizing the effect of dose the antigens used to mediate desired immune response and protection.

Further, the immunization with 6kDa antigen demonstrated a significant percent survival which was better than that observed in BCG vaccinated mice (70%) and 45% in control groups, challenged at the peak of immune response with LD_{50} of *M. tuberculosis* $H_{37}Rv$.

These findings are further substantiated by the earlier reports where in the protective effect of the secretory proteins has been demonstrated to be comparable to BCG vaccine.

Thus our results also support the emerging hypothesis that secretory proteins are the key immnuoprotective molecules capable of generating a strong cell mediated immune response leading to an efficient acquired resistance against virulent *M. tuberculosis* H_{37}Rv and other intracellular pathogens.

Further, studies were conducted to evaluate the immnuoprotective behavior of 6kDa secretory protein using liposome as adjuvant.

Immunization with 6kDa antigen encapsulated in liposomes, included significant humoral and cell mediated immune responses as determined by ELISA. The augmentation of T and B lymphocyte mediated immune response is in accordance with earlier reports in which liposomes have been used as adjuvant for *Mycobacterial* antigens

The immune response (cellular and humoral) elicited by the 6kDa secretory protein entrapped in liposomes were similar to that elicited by 6 kDa-FIA and BCG vaccine, thus indicating that liposomes can be effectively used to generate strong immune response by the entrapped antigen.

Immunization with 6kDa secretory protein entrapped in liposomes resulted in increased resistance to intravenous challenge with LD_{50} of *M. tuberculosis*

$H_{37}Rv$ as immunized animals showed considerably higher ($p<0.001$) survival rates (76%) as a compared to control animals (32%) and BCG vaccinated animals (72%) on 30 days post challenge.

There was a generalized drop in the number of bacteria isolated from spleen, lungs and liver as compared to control mice indicated that immunization with 6kDa-Lip ensure better clearance of the microorganism from the three organs (liver, lungs and spleen).

Our findings that liposomes could be effectively used to elicit the desired immune response and confer significant protection in the immunized animals are similar to the observation of other workers who have demonstrated increased immune response with the liposomal encapsulated antigen.

Thus, the results of the present study strongly suggest that the secretory proteins of *M. tuberculosis* are immunogenic and induce strong humoral and cellular responses.

Further, it was also observed that the immune response induced by some of these antigens is strongly humoral and that by other antigens are cellular. A careful and selective selection of these antigens can by useful in development of an effective diagnostic and a prophylactic tool.

8

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9

Appendix

Results of Antibody detection (Cocktail of Secretory Protein 'Ag' with ELISA)

Table-I

Evaluation of Cocktail of 'Secretory Protein Ag' with ELISA test-

S. No.	Group	No. of Serum sample	Cocktail of Secretory protein Ag, ELISA Test		Reactivity
			+ Ve	-Ve	
1	Pulmonary tuberculosis	148	139	09	94.27 %
2	Extra-pulmonary tuberculosis	50	46	04	92.59%
3	Meningitis tuberculosis	14	05	09	60.86%
4	Malaria	18	00	18	Non-reactive
5	Leprosy	25	02	23	92.5% Specificity
6	Hepatitis B/C	11	01	10	Cross-reactive
7	Lung cancer	32	2	30	Cross-reactive
8	Bacterial pneumonia	18	1	17	Cross-reactive
9	Normal healthy	30	30	30	Non-reactive
10	BCG Vaccinated	58	01	57	Cross-reactive
11	BCG Unvaccinated (Normal sample)	27	2	25	Cross-reactive

Evaluation study: Department of Microbiology& Immunology, Gandhi Medical College Bhopal, M.P.India

Table-II

Study with TB sera

Type of infection-Pulmonary tuberculosis

Sr. No.	Code No. of patients	Smear status at the time of beginning	Clinical history	Smear status at the time of sampling	Duration of therapy	Results CSP Ag, ELISA test	Reactivity
1	28	+ve	AFB +ve	+ve	15	+ve	Reactive
2	14	+ve	AFB +ve	+ve	20	+ve	Reactive
3	44	+ve	AFB +ve	+ve	20	+ve	Reactive
4	11	+ve	X-ray	+ve	45	+ve	Reactive
5	32	+ve	X-ray	+ve	05	+ve	Reactive
6	38	+ve	X-ray	+ve	10	+ve	Reactive
7	37	+ve	X-ray	+ve	45	+ve	Reactive
8	39	+ve	X-ray	+ve	15	+ve	Reactive
9	29	+ve	X-ray	+ve	05	+ve	Reactive
10	05	+ve	X-ray	+ve	03	+ve	Reactive
11	03	+ve	X-ray	+ve	90	+ve	Reactive
12	40	+ve	AFB +ve	+ve	15	+ve	Reactive
13	102	+ve	AFB +ve	+ve	30	+ve	Reactive
14	18	+ve	AFB +ve	+ve	30	+ve	Reactive
15	165	+ve	AFB +ve	+ve	45	+ve	Reactive
16	143	+ve	AFB +ve	+ve	08	+ve	Reactive
17	123	+ve	AFB +ve	+ve	60	+ve	Reactive
18	153	+ve	AFB +ve	+ve	75	+ve	Reactive
19	178	+ve	AFB +ve	+ve	30	+ve	Reactive
20	156	+ve	AFB +ve	+ve	30	+ve	Reactive
21	109	+ve	AFB +ve	+ve	15	-ve	Non-reactive
22	GM-02	+ve	AFB +ve	+ve	12	+ve	Reactive
23	GM-6	+ve	AFB +ve	+ve	45	+ve	Reactive
24	GM-23	+ve	X-ray	+ve	20	+ve	Reactive
25	GM-27	+ve	X-ray	+ve	45	+ve	Reactive
26	GM-32	+ve	X-ray	+ve	30	+ve	Reactive
27	GM36	+ve	X-ray	+ve	30	+ve	Reactive
28	GM-40	+ve	X-ray	+ve	15	+ve	Reactive
29	GM-43	+ve	X-ray	+ve	12	+ve	Reactive
30	GM-44	+ve	X-ray	+ve	45	+ve	Reactive
31	GM-45	+ve	X-ray	+ve	20	+ve	Reactive
32	GM-48	+ve	X-ray	+ve	10	-ve	Non-reactive
33	GM-50	+ve	X-ray	+ve	30	+ve	Reactive
34	GM-53	+ve	X-ray	+ve	40	+ve	Reactive
35	GM-60	+ve	X-ray	+ve	16	+ve	Reactive
36	GM-62	+ve	X-ray	+ve	30	+ve	Reactive
37	GM-63	+ve	X-ray	+ve	26	+ve	Reactive
38	GM-68	+ve	AFB +ve	+ve	45	+ve	Reactive
39	GM-69	+ve	AFB +ve	+ve	06	-ve	Non-reactive

40	GM-72	+ve	X-ray	+ve	40	+ve	Reactive
41	GM-75	+ve	AFB +ve	+ve	03	+ve	Reactive
42	GM-74	+ve	AFB +ve	+ve	02	+ve	Reactive
43	GM-55	+ve	Clinically	+ve	72	+ve	Reactive
44	GM-78	+ve	X-ray	+ve	14	+ve	Reactive
45	GM-81	+ve	Clinically	+ve	43	+ve	Reactive
46	GM-83	+ve	Clinically	+ve	30	-ve	Non-reactive
47	GM-84	+ve	Clinically	+ve	20	+ve	Reactive
48	GM-87	+ve	Clinically	+ve	40	+ve	Reactive
49	GM-90	+ve	AFB +Ve	+ve	13	+ve	Reactive
50	GM-08	+ve	Clinically	+ve	20	+ve	Reactive
51	105	+ve	Clinically	+ve	40	+ve	Reactive
52	110	+ve	AFB +ve	+ve	13	+ve	Reactive
53	112	+ve	Kp-90	+ve	20	+ve	Reactive
54	114	+ve	AFB +ve	+ve	45	+ve	Reactive
55	119	+ve	AFB +ve	+ve	30	+ve	Reactive
56	10	+ve	Clinically	+ve	30	+ve	Reactive
57	22	+ve	Clinically	+ve	30	+ve	Reactive
58	34	+ve	Clinically	+ve	08	+ve	Reactive
59	74	+ve	Clinically	+ve	08	+ve	Reactive
60	28	+ve	Clinically	+ve	20	+ve	Reactive
61	14	+ve	AFB +ve	+ve	02	+ve	Reactive
62	44	+ve	AFB/X-ray	+ve	35	+ve	Reactive
63	11	+ve	X-ray	+ve	30	+ve	Reactive
64	32	+ve	X-ray	+ve	40	+ve	Reactive
65	38	+ve	X-ray	+ve	23	+ve	Reactive
66	37	+ve	X-ray	+ve	60	-ve	Non-reactive
67	39	+ve	X-ray	+ve	45	+ve	Reactive
68	29	+ve	X-ray	+ve	10	+ve	Reactive
69	L-25	+ve	X-ray	+ve	10	+ve	Reactive
70	W-06	+ve	X-ray	+ve	05	+ve	Reactive
71	W-04	+ve	X-ray	+ve	20	+ve	Reactive
72	W-05	+ve	X-ray	+ve	10	+ve	Reactive
73	Sonia	+ve	X-ray	+ve	45	+ve	Reactive
74	W-28	+ve	X-ray	+ve	15	+ve	Reactive
75	W-01	+ve	X-ray	+ve	30	+ve	Reactive
76	W-02	+ve	Clinically	+ve	10	+ve	Reactive
77	W-03	+ve	Clinically	+ve	40	+ve	Reactive
78	W-08	+ve	Clinically	+ve	15	+ve	Reactive
79	M/2/10	+ve	Clinically	+ve	30	+ve	Reactive
80	R-1/05	+ve	Clinically	+ve	30	+ve	Reactive
81	R-1/06	+ve	AFB +ve	+ve	60	+ve	Reactive
82	R-1/8	+ve	AFB +ve	+ve	10	+ve	Reactive
83	R-1/12	+ve	Clinically	+ve	15	+ve	Reactive
84	R-1/15	+ve	AFB +ve/ X-ray	+ve	02	+ve	Reactive
85	R-1/20	+ve	AFB +ve/ X-ray	+ve	45	+ve	Reactive

86	R-1/24	+ve	AFB +ve/ X-ray	+ve	45	+ve	Reactive
87	R-1/33	+ve	Clinically	+ve	10	+ve	Reactive
88	R-1/35	+ve	Clinically	+ve	38	+ve	Reactive
89	R-1/38	+ve	Clinically	+ve	10	-ve	Non-reactive
90	R-1/44	+ve	Clinically	+ve	06	+ve	Reactive
91	R-1/51	+ve	Clinically	+ve	20	+ve	Reactive
92	R-1/10	+ve	X-ray	+ve	12	+ve	Reactive
93	R-1/54	+ve	X-ray/AFB	+ve	05	+ve	Reactive
94	R-2/2	+ve	KP-90	+ve	10	+ve	Reactive
95	R-2/4	+ve	Kp-90	+ve	20	+ve	Reactive
96	R-2/5	+ve	LAM	+ve	95	-ve	Non-reactive
97	R-2/8	+ve	X-ray/AFB	+ve	04	+ve	Reactive
98	R-2/12	+ve	LAM	+ve	46	+ve	Reactive
99	R-2/14	+ve	Clinically	+ve	30	+ve	Reactive
100	R-2/18	+ve	Clinically	+ve	20	-ve	Non-reactive
101	W-2/3	+ve	Clinically	+ve	10	+ve	Reactive
102	W-2/6	+ve	Clinically	+ve	04	+ve	Reactive
103	W-2/7	+ve	Clinically	+ve	25	+ve	Reactive
104	W-2/12	+ve	Clinically	-ve	62	+ve	Reactive
105	W-2/14	+ve	AFB/X-ray	+ve	12	+ve	Reactive
106	W-2/18	+ve	ABF/X-ray	+ve	09	+ve	Reactive
107	W-2/20	+ve	ABF/X-ray	+ve	06	+ve	Reactive
108	W-2 /24	+ve	ABF/X-ray	+ve	14	+ve	Reactive
109	W-2/27	+ve	X-ray	+ve	16	+ve	Reactive
110	W-2/29	+ve	X-ray	+ve	23	+ve	Reactive
111	W-2/32	+ve	X-ray	+ve	22	+ve	Reactive
112	W-2/38	+ve	X-ray	+ve	26	+ve	Reactive
113	Amin Chand	+ve	AFB +ve	+ve	04	+ve	Reactive
114	Deepa	+ve	AFB +ve	+ve	02	+ve	Reactive
115	Madan	+ve	AFB +ve	+ve	01	+ve	Reactive
116	Roopa	+ve	AFB +ve	+ve	00	+ve	Reactive
117	Nerendra	+ve	AFB +ve	+ve	06	+ve	Reactive
118	Kala Bai	+ve	AFB +ve	+ve	10	+ve	Reactive
119	Naseen Bai	+ve	AFB +ve	+ve	00	+ve	Reactive
120	Sahida Bai	+ve	AFB +ve	+ve	07	+ve	Reactive
121	Rekha	+ve	X-ray	+ve	02	+ve	Reactive
122	Punia	+ve	AFB +ve	+ve	09	+ve	Reactive
123	Seema	+ve	AFB +ve	+ve	21	+ve	Reactive
124	Ram Gopal	+ve	AFB +ve	+ve	16	+ve	Reactive
125	Dasarath	+ve	AFB +ve	+ve	02	+ve	Reactive
126	Nand Kishor	+ve	AFB +ve	+ve	28	+ve	Reactive
127	Dherendra Singh	+ve	X-ray	+ve	00	+ve	Reactive
128	Harprasad	+ve	AFB +ve	-ve	00	+ve	Reactive
129	Abdul	+ve	AFB +ve	+ve	08	+ve	Reactive
130	Shaelendra	+ve	AFB +ve	+ve	02	+ve	Reactive
131	Hushain	+ve	AFB +ve	+ve	20	+ve	Reactive
132	Kamal Singh	+ve	X-ray/AFB	+ve	04	+ve	Reactive

133	J.P.Srivastava	+ve	X-ray/AFB	+ve	06	+ve	Reactive
134	Babulal	+ve	X-ray	+ve	02	+ve	Reactive
135	Girja Bai	+ve	X-ray	+ve	10	+ve	Reactive
136	Anup Singh	+ve	X-ray	+ve	30	+ve	Reactive
137	Amrit Lal	+ve	AFB +ve	+ve	00	+ve	Reactive
138	Prabhu Das	+ve	AFB +ve	+ve	22	+ve	Reactive
139	Dannu	+ve	AFB +ve	+ve	01	+ve	Reactive
140	Yugendra Mishr	+ve	AFB/X-ray	+ve	01	+ve	Reactive
141	Amshi Bai	+ve	AFB +ve	+ve	03	-ve	Non-reactive
142	Kamta Prasad	+ve	AFB +ve	+ve	02	+ve	Reactive
143	Rani Pandey	+ve	X-ray/AFB	+ve	08	+ve	Reactive
144	Alka Bai	+ve	AFB +ve	+ve	05	+ve	Reactive
145	Gyan Singh	+ve	AFB +ve	+ve	08	+ve	Reactive
146	Badri Prasad	+ve	AFB +ve	+ve	02	+ve	Reactive
147	Baseen Khan	+ve	AFB +ve	+ve	02	+ve	Reactive
148	Mahesh Kumar	+ve	AFB +ve	+ve	14	+ve	Reactive

Non-reactive sample:09
Total Sample tested: 148, (pulmonary tuberculosis)
Sensitivity: TP/TP+FNX100=148/148+09=148/157X100=94.27 %

Table-III

Evaluation study with Extra-pulmonary tuberculosis: Collected from GTB Hospital Delhi-7

Sr. No.	Code No. of patients	Clinically conformed	Clinical investigation as per record	Results of CSP Ag, ELISA test	Reactivity
1	70	LAM test	Extrapulmonary +ve	+ve	Reactive
2	L-25	LAM	Extrapulmonary +ve	+ve	Reactive
3	W-06	LAM	Extrapulmonary +ve	+ve	Reactive
4	W-04	LAM	Extrapulmonary +ve	+ve	Reactive
5	W-05	LAM	Extrapulmonary +ve	+ve	Reactive
6	Prasad	LAM	Extrapulmonary +ve	+ve	Reactive
7	W-28	LAM	Extrapulmonary +ve	+ve	Reactive
8	W-01	LAM	Extrapulmonary +ve	+ve	Reactive

9	W-02	LAM	Extrapulmonary +ve	+ve	Reactive
10	W-03	LAM	Extrapulmonary +ve	+ve	Reactive
11	W-08	LAM	Extrapulmonary +ve	+ve	Reactive
12	M/2/10	Clinically	Extrapulmonary +ve	-ve	Non-reactive
13	R-1/05	Clinically	Extrapulmonary +ve	+ve	Reactive
14	R-1/05	KP-90/LAM	Extrapulmonary +ve	+ve	Reactive
15	M-1/05	KP-90/LAM	Extrapulmonary +ve	+ve	Reactive
16	M-1/29	KP-90/LAM	Extrapulmonary +ve	+ve	Reactive
17	M-1/07	KP-90/LAM	Extrapulmonary +ve	+ve	Reactive
18	M-2/04	KP-90/LAM	Extrapulmonary +ve	+ve	Reactive
19	M-2/06	KP-90/LAM	Extrapulmonary +ve	-ve	Non-reactive
20	M-2/07	KP-90/LAM	Extrapulmonary +ve	+ve	Reactive
21	M-2/012	KP-90/LAM	Extrapulmonary +ve	+ve	Reactive
22	M-2/22	KP-90/LAM	Extrapulmonary +ve	+ve	Reactive
23	M-2/25	LAM	Extrapulmonary +ve	+ve	Reactive
24	Phool Bai	LAM	Extrapulmonary +ve	+ve	Reactive
25	Ashok	LAM	Extrapulmonary +ve	+ve	Reactive
26	Neelam	LAM	Extrapulmonary +ve	+ve	Reactive
27	Raju	LAM	Extrapulmonary +ve	+ve	Reactive
28	Raghubir Singh	LAM	Extrapulmonary +ve	+ve	Reactive
29	Farida	KP-90	Extrapulmonary +ve	+ve	Reactive
30	GM-03	Clinically	Extrapulmonary +ve	+ve	Reactive
31	GM-05	Clinically	Extrapulmonary +ve	+ve	Non-reactive
32	GM-07	Clinically	Extrapulmonary +ve	+ve	Reactive
33	GM-08	Clinically	Extrapulmonary +ve	+ve	Reactive
34	GM-11	Clinically	Extrapulmonary +ve	+ve	Reactive
35	GM-20	Clinically	Extrapulmonary +ve	+ve	Reactive

36	GM-29	Clinically	Extrapulmonary +ve	+ve	Reactive
37	GM-42	Clinically	Extrapulmonary +ve	+ve	Reactive
38	GM-45	Clinically	Extrapulmonary +ve	+ve	Reactive
39	GM-49	Clinically	Extrapulmonary +ve	+ve	Reactive
40	GM-52	LAM	Extrapulmonary +ve	+ve	Reactive
41	GM-57	Clinically	Extrapulmonary +ve	-ve	Non-reactive
42	GM-61	Clinically	Extrapulmonary +ve	+ve	Reactive
43	GM-63	Clinically	Extrapulmonary +ve	+ve	Reactive
44	GM-64	LAM	Extrapulmonary +ve	+ve	Reactive
45	GM-86	LAM	Extrapulmonary +ve	+ve	Reactive
46	GM-90	LAM	Extrapulmonary +ve	+ve	Reactive
47	GM-103	LAM	Extrapulmonary +ve	+ve	Reactive
48	GM-108	LAM	Extrapulmonary +ve	+ve	Reactive
49	GM-115	Clinically	Extrapulmonary +ve	+ve	Reactive
50	GM-123	Clinically	Extrapulmonary +ve	+ve	Reactive

Non-reactive samples=04
Total no. of sample tested: - 50,
Sensitivity: 50/50+4=50/54x100= 92.59 %.

Table-IV

Cross reactivity Study of 'Cocktail of Secretory Protein Ag' ELISA test with Leprosy sera. Sample collected from JALMA Institute of leprosy, Agra. India.

Sr. No.	Code No. of patients	Clinical history	Clinical investigation as per record	Results, CSP Ag, ELISA test	Reactivity	Remark
1	07	Clinically +ve	Leprosy +ve	-ve	Non-reactive	OPD
2	06	Clinically +ve	Leprosy +ve	-ve	Non-reactive	OPD
3	92CT	Clinically +ve	Leprosy +ve	-ve	Non-reactive	OPD
4	22	Clinically +ve	Leprosy +ve	-ve	Non-reactive	OPD
5	34	Clinically +ve	Leprosy +ve	-ve	Non-reactive	OPD
6	74	Clinically +ve	Leprosy +ve	-ve	Non-reactive	OPD
7	28	Clinically +ve	Leprosy +ve	-ve	Non-reactive	OPD
8	14	Clinically +ve	Leprosy +ve	+ve	Cross-reactive	OPD
9	44	Clinically +ve	Leprosy +ve	-ve	Non-reactive	OPD
10	11	Clinically +ve	Leprosy +ve	-ve	Non-reactive	OPD

11	32	Clinically +ve	Leprosy +ve	-ve	Non-reactive	OPD
12	38	Clinically +ve	Leprosy +ve	-ve	Non-reactive	OPD
13	37	Clinically +ve	Leprosy +ve	-ve	Non-reactive	OPD
14	39	Clinically +ve	Leprosy +ve	-ve	Non-reactive	OPD
15	29	Clinically +ve	Leprosy +ve	-ve	Non-reactive	OPD
16	05	Clinically +ve	Leprosy +ve	-ve	Non-reactive	OPD
17	03	Clinically +ve	Leprosy +ve	-ve	Non-reactive	OPD
18	06	Clinically +ve	Leprosy +ve	-ve	Non-reactive	OPD
19	12	Clinically +ve	Leprosy +ve	-ve	Non-reactive	OPD
20	10	Clinically +ve	Leprosy +ve	+ve	Cross-reactive	OPD
21	07	Clinically +ve	Leprosy +ve	-ve	Non-reactive	OPD
22	01	Clinically +ve	Leprosy +ve	-ve	Non-reactive	OPD
23	14	Clinically +ve	Leprosy +ve	-ve	Non-reactive	OPD
24	08	Clinically +ve	Leprosy +ve	-ve	Non-reactive	OPD
25	09	Clinically +ve	Leprosy +ve	-ve	Non-reactive	OPD

False positive=02

Specificity=TN/TN+FP=25/25+2=25/27x100=92.59%

Table-V

Evaluation study with Tubercle Meningitis sera. Collected from S.S. Medical College Rewa, M.P., India.

Type of infection: Meningitis tuberculosis

Sr. No.	Code no. of patients	Clinical confirmation	Result of CSP 'Ag' ELISA test	Reactivity
1	GM-2/02	Symptoms	+ve	Reactive
2	GM-2/05	Symptoms	-ve	Non-reactive
3	GM-2/ 06	Symptoms	+ve	Reactive
4	GM-2/12	Symptoms	-ve	Non-reactive
5	GM-3/03	Symptoms	-ve	Non-reactive
6	GM-3/08	Symptoms	-ve	Non-reactive
7	GM-3/11	Symptoms	+ve	Reactive
8	GM-3/14	Symptoms	+ve	Non-reactive
9	GM-3/16	Symptoms	+ve	Non-reactive
10	GM-3/22	Symptoms	-ve	Non-reactive
11	GM-3/26	Symptoms	-ve	Non-reactive
12	GM-2/16	Symptoms	+ve	Reactive
13	GM-3/27	Symptoms	+ve	Reactive
14	GM-2/03	Symptoms	-ve	Non-reactive

Sensitivity=TP/TP+FNX100=14/14+9=14/23X100=60.86%

Study with other common infection group. Samples Collected from G.M.C. Bhopal M.P. India.

Table-VI

Sr. No.	Clinical status of specimen	Clinically confirmation	No. of sample tested	Results of CSP Ag, ELISA test		Reactivity
				+ve	-ve	
1	Lung cancer	Symptoms	32	01	31	Cross-reactive
2	Malaria	Slide test	18	00	18	Non-reactive
3	Hepatitis-B/C	Abbt.	16	00	16	Non-reactive
4	Bacterial pneumonia	Gram staining	18	00	18	Non-reactivity
5	Pulmonary aspergillosis	Clinical	15	01	14	Cross-reactive

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Publications

- Garg S. K., R. P Tiwari, **Dileep Tiwari,** R.Singh, D. Malhotra, V. K. Ramnani. G. B. K. S. Prasad, R. Chandra, M. Fraziano, V. Colizzi and P. S. Bisen. 2003.Diagnosis of Tuberculosis: Available Technologies, Limitations and Possibilities. J. Clin. Lab Anal. 16:1-7.

- Tiwari R. P., **Dileep Tiwari**, S. K. Garg, R. Chandra, P. S. Bisen, 2005. Glycolipids of *M.tuberculosis* strain $H_{37}Rv$ are Potential serological markers for diagnosis of active tuberculosis. J.Clin. Diagnostic Lab. Immunology.12; 3: 465-473.

- Tiwari R. P., **Dileep Tiwari**, S. K. Singh and P. S. Bisen.2006. Development of a rapid liposomal agglutination card test for the detection of antigen in patients with Meningial, Pulmonary and other Extra-pulmonary tuberculosis. -Communicated.

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